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- (74) Agent: STUART, Donald, R.; Dow AgroSciences LLC, 9330 Zionsville Road, Indianapolis, IN 46268 (US).
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- (71) Applicant: DOW AGROSCIENCES LLC [US/US]; 9330 Zionsville Road, Indianapolis, IN 46268 (US).
- (72) Inventors: PETELL, James, K.; 16825 Meyer Lane, Grass Valley, CA 95949 (US). MERLO, Donald, J.; 11845 Durbin Drive, Carmel, IN 46032 (US). HERMAN, Rod, A.; 11153 West 500 South, New Ross, IN 47968 (US). ROBERTS, Jean, L.; 26035 State Road 19, Arcadia, IN 46030 (US). GUO, Lining; 3212 Summit Ridge Loop, Morrisville, NC 27560 (US). SCHAFER, Barry, W.; 1429 Lighthouse Point, Cicero, IN 46034 (US). SUKHAPINDA, Kitisri; 4748 Ashwood Court, Zionsville, IN 46077 (US). MERLO, Ann, Owens; 11845 Durbin Drive, Carmel, IN 46032 (US).
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(54) Title: TRANSGENIC PLANTS EXPRESSING PHOTORHABDUS TOXIN

(57) Abstract: Novel polynucleotide sequences that encode insect toxins TcdA and TcbA have base compositions that differ substantially from the native genes, making them more similar to plant genes. The new sequences are suitable for use for high expression in both monocots and dicots. Transgenic plants with a genome comprising a nucleic acid of SEQ ID NO: 3 or SEQ ID NO:4 are insect resistant.

TRANSGENIC PLANTS EXPRESSING PHOTORHABDUS TOXIN

BACKGROUND OF THE INVENTION

As reported in WO98/08932, protein toxins from the genus *Photorhabdus* have been shown to have oral toxicity against insects. The toxin complex produced by *Photorhabdus luminescens* (W-14), for example, has been shown to contain ten to fourteen proteins, and it is known that these are produced by expression of genes from four distinct genomic regions: *tca*, *tcb*, *tcc*, and *tcd*. WO98/08932 discloses nucleotide sequences for the native toxin genes.

Of the separate toxins isolated from *Photorhabdus luminescens* (W-14), those designated Toxin A and Toxin B are especially potent against target insect species of interest, for example corn rootworm. Toxin A is comprised of two different subunits. The native gene *tcdA* (SEQ ID NO:1) encodes protoxin TcdA (see SEQ ID NO:1). As determined by mass spectrometry, TcdA is processed by one or more proteases to provide Toxin A. More specifically, TcdA is an approximately 282.9 kDA protein (2516 aa) that is processed to provide TcdAii, an approximately 208.2 kDA (1849 aa) protein encoded by nucleotides 265-5811 of SEQ ID NO:1, and TcdAiii, an approximately 63.5 kDA (579 aa) protein encoded by nucleotides 5812-7551 of SEQ ID NO:1.

Toxin B is similarly comprised of two different subunits. The native gene *tcbA* (SEQ ID NO:2) encodes protoxin TcbA (see SEQ ID NO:2). As determined by mass spectrometry, TcbA is processed by one or more proteases to provide Toxin B. More specifically, TcbA is an approximately 280.6 kDA (2504 aa) protein that is processed to provide TcbAii, an approximately 207.7 kDA (1844 aa) protein encoded by nucleotides 262-5793 of SEQ ID NO:2 and TcbAiii, an approximately 62.9 kDA (573 aa) protein encoded by nucleotides 5794-7512 of SEQ ID NO:2.

The native *tcdA* and *tcbA* genes are not well suited for high level expression in plants. They encode multiple destabilization sequences, mRNA splice sites, polyA addition sites and other possibly detrimental sequence motifs. In addition, the codon compositions are not like those of plant genes. W098/08932 gives general guidance on how the toxin genes could be reengineered to more efficiently expressed in the cytoplasm of plants, and describes how plants can be transformed to incorporate the *Phototrhabdus* toxin genes into their genomes.

SUMMARY OF THE INVENTION

In a preferred embodiment, the invention provides novel polynucleotide sequences that encode TcdA and TcbA. The novel sequences have base compositions that differ substantially from the native genes, making them more similar to plant genes. The new sequences are suitable for use for high expression in both monocots and dicots, and this feature is designated by referring to the sequences as the "hemicot" criteria, which is set forth in detail hereinafter. Other important features of the sequences are that potentially deleterious sequences have been eliminated, and unique restriction sites have been built in to enable adding or changing expression elements, organellar targeting signals, engineered protease sites and the like, if desired.

In a particularly preferred embodiment, the invention provides polynucleotide sequences that satisfy hemicot criteria and that comprise a sequence encoding an endoplasmic reticulum signal or similar targeting sequence for a cellular organelle in combination with a sequence encoding TcdA or TdbA.

More broadly, the invention provides engineered nucleic acids encoding functional *Phototrhabdus* toxins wherein the sequences satisfy hemicot criteria.

The invention also provides transgenic plants with genomes comprising a novel sequence of the invention that imparts functional activity against insects.

5 BRIEF DESCRIPTION OF SEQUENCES

SEQ ID NO:1 is the native *tcdA* DNA sequence together with the corresponding encoded amino acid sequence for TcdA.

10 SEQ ID NO:2 is the native *tcbA* DNA sequence together with the corresponding encoded amino acid sequence for TcbA.

SEQ ID NO:3 is an artificial sequence encoding TcdA that is suitable for expression in monocot and dicot plants.

15 SEQ ID NO:4 is an artificial sequence encoding TdbA that is suitable for expression in monocot and dicot plants.

SEQ ID NO:5 is an artificial hemicot sequence that encodes the 21 amino acid ER signal peptide of 15 kDa
20 zein from Black Mexican Sweet maize.

SEQ ID NO:6 is an artificial hemicot sequence that encodes for the full-length native TcdA protein (amino acids 22-2537) fused to the modified 15 kDa zein endoplasmic reticulum signal peptide (amino acids 1-21).

25 DETAILED DESCRIPTION

The native *Photobacterium* toxins are protein complexes that are produced and secreted by growing bacteria cells of the genus *Photobacterium*. Of particular interest are the proteins produced by the species *Photobacterium*
30 *luminescens*. The protein complexes have a molecular size of approximately 1,000 kDa and can be separated by SDS-PAGE gel analysis into numerous component proteins. The toxins contain no hemolysin, lipase, type C phospholipase, or nuclease activities. The toxins
35 exhibit significant toxicity upon ingestion by a number of insects.

A unique feature of *Photorhabdus* is its bioluminescence. *Photorhabdus* may be isolated from a variety of sources. One such source is nematodes, more particularly nematodes of the genus *Heterorhabditis*.

- 5 Another such source is from human clinical samples from wounds, see Farmer et al. 1989 J. Clin. Microbiol. 27 pp. 1594-1600. These saprophytic strains are deposited in the American Type Culture Collection (Rockville, MD) ATCC #s 43948, 43949, 43950, 43951, and 43952, and are
10 incorporated herein by reference. It is possible that other sources could harbor *Photorhabdus* bacteria that produce insecticidal toxins. Such sources in the environment could be either terrestrial or aquatic based.

- The genus *Photorhabdus* is taxonomically defined as a
15 member of the Family *Enterobacteriaceae*, although it has certain traits atypical of this family. For example, strains of this genus are nitrate reduction negative, yellow and red pigment producing and bioluminescent. This latter trait is otherwise unknown within the
20 *Enterobacteriaceae*. *Photorhabdus* has only recently been described as a genus separate from the *Xenorhabdus* (Boemare et al., 1993 Int. J. Syst. Bacteriol. 43, 249-255). This differentiation is based on DNA-DNA hybridization studies, phenotypic differences (e.g.,
25 presence (*Photorhabdus*) or absence (*Xenorhabdus*) of catalase and bioluminescence) and the Family of the nematode host (*Xenorhabdus*; *Steinernematidae*, *Photorhabdus*; *Heterorhabditidae*). Comparative, cellular fatty-acid analyses (Janse et al. 1990, Lett. Appl. Microbiol 10, 131-135; Suzuki et al. 1990, J. Gen. Appl. Microbiol., 36, 393-401) support the separation of
30 *Photorhabdus* from *Xenorhabdus*.

- Currently, the bacterial genus *Photorhabdus* is comprised of a single defined species, *Photorhabdus*
35 *luminescens* (ATCC Type strain #29999, Poinar et al., 1977, Nematologica 23, 97-102). A variety of related

strains have been described in the literature (e.g.,
Akhurst et al. 1988 J. Gen. Microbiol., 134, 1835-1845;
Boemare et al. 1993 Int. J. Syst. Bacteriol. 43 pp. 249-
255; Putz et al. 1990, Appl. Environ. Microbiol., 56,
5 181-186).

The following toxin producing *Photorhabdus* strains
have been deposited:

strain	accession number	date of deposit
W-14	ATCC 55397	March 5, 1993
WX1	NRRL B-21710	April 29, 1997
WX2	NRRL B-21711	April 29, 1997
WX3	NRRL B-21712	April 29, 1997
WX4	NRRL B-21713	April 29, 1997
WX5	NRRL B-21714	April 29, 1997
WX6	NRRL B-21715	April 29, 1997
WX7	NRRL B-21716	April 29, 1997
WX8	NRRL B-21717	April 29, 1997
WX9	NRRL B-21718	April 29, 1997
WX10	NRRL B-21719	April 29, 1997
WX11	NRRL B-21720	April 29, 1997
WX12	NRRL B-21721	April 29, 1997
WX14	NRRL B-21722	April 29, 1997
WX15	NRRL B-21723	April 29, 1997
H9	NRRL B-21727	April 29, 1997
Hb	NRRL B-21726	April 29, 1997
Hm	NRRL B-21725	April 29, 1997
HP88	NRRL B-21724	April 29, 1997
NC-1	NRRL B-21728	April 29, 1997
W30	NRRL B-21729	April 29, 1997
WIR	NRRL B-21730	April 29, 1997
B2	NRRL B-21731	April 29, 1997
ATCC 43948	ATCC 55878	November 5, 1996
ATCC 43949	ATCC 55879	November 5, 1996
ATCC 43950	ATCC 55880	November 5, 1996
ATCC 53951	ATCC 55881	November 5, 1996
ATCC 43952	ATCC 55882	November 5, 1996
DEPI	NRRL B-21707	April 29, 1997
DEP2	NRRL B-21708	April 29, 1997
DEP3	NRRL B-21709	April 29, 1997
<i>P. zealandrica</i>	NRRL B-21683	April 29, 1997
<i>P. hepialus</i>	NRRL B-21684	April 29, 1997
HB-Arg	NRRL B-21685	April 29, 1997
HB Oswego	NRRL B-21686	April 29, 1997
Hb Lewiston	NRRL B-21687	April 29, 1997
K-122	NRRL B-21688	April 29, 1997
HMGD	NRRL B-21689	April 29, 1997
Indicus	NRRL B-21690	April 29, 1997
GD	NRRL B-21691	April 29, 1997
PWH-5	NRRL B-21692	April 29, 1997
Megidis	NRRL B-21693	April 29, 1997
HF-85	NRRL B-21694	April 29, 1997
A. Cows	NRRL B-21695	April 29, 1997
MP1	NRRL B-21696	April 29, 1997
MP2	NRRL B-21697	April 29, 1997
MP3	NRRL B-21698	April 29, 1997
MP4	NRRL B-21699	April 29, 1997
MP5	NRRL B-21700	April 29, 1997
GL98	NRRL B-21701	April 29, 1997
GL101	NRRL B-21702	April 29, 1997
GL138	NRRL B-21703	April 29, 1997
GL155	NRRL B-21704	April 29, 1997
GL217	NRRL B-21705	April 29, 1997
GL257	NRRL B-21706	April 29, 1997

All strains were deposited in accordance with the terms of the Budapest Treaty. Strains having

accession numbers prefaced by "ATTC" were deposited on the indicated date in the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852 USA. Strains prefaced by "NRRL" were
5 deposited on the indicated date in the Agricultural Research Service Patent Culture Collection (NRRL), National Center for Agricultural Utilization Research, ARS-USDA, 1815 North University St., Peoria IL 61604 USA.

10 The present invention provides hemicot nucleic acid sequences encoding toxins from any *Photorhabdus* species or strain that produces a toxin having functional activity. Hemicot nucleic acid sequences encoding proteins homologous to such toxins are also encompassed
15 by the invention.

Several terms that are used herein have a particular meaning and are defined as follows:

By "functional activity" it is meant herein that the protein toxins) function as insect control agents in that
20 the proteins are orally active, or have a toxic effect, or are able to disrupt or deter feeding, which may or may not cause death of the insect. When an insect comes into contact with an effective amount of toxin delivered via transgenic plant expression, formulated protein
25 compositions), sprayable protein compositions), a bait matrix or other delivery system, the results are typically death of the insect, or the insects do not feed upon the source which makes the toxins available to the insects.

30 By "homolog" it is meant an amino acid sequence that is identified as possessing homology to a reference *Photorhabdus* toxin polypeptide amino acid sequence.

By "homology" it is meant an amino acid sequence that has a similarity index of at least 33% and/or an
35 identity index of at least 26% to a reference *Photorhabdus* toxin polypeptide amino acid sequence, as

scored by the GAP algorithm using the B10sum 62 protein scoring matrix Wisconsin Package Version 9.0, Genetics Computer Group GCG), Madison, WI).

By "identity" is meant an amino acid sequence that
5 contains an identical residue at a given position,
following alignment with a reference *Photorhabdus* toxin
polypeptide amino acid sequence by the GAP algorithm.

By the use of the term "*Photorhabdus* toxin" it is
meant any protein produced by a *Photorhabdus*
10 microorganism strain which has functional activity
against insects, where the *Photorhabdus* toxin could be
formulated as a sprayable composition, expressed by a
transgenic plant, formulated as a bait matrix, delivered
via baculovirus, or delivered by any other applicable
15 host or delivery system.

By the use of the term "toxic" or "toxicity" as used
herein it is meant that the toxins produced by
Photorhabdus have "functional activity" as defined
herein.

By "substantial sequence homology" is meant either:
a DNA fragment having a nucleotide sequence sufficiently
similar to another DNA fragment to produce a protein
having similar biochemical properties; or a polypeptide
having an amino acid sequence sufficiently similar to
25 another polypeptide to exhibit similar biochemical
properties.

As with other bacterial toxins, the rate of mutation
of the bacteria in a population causes many related
toxins slightly different in sequence to exist. Toxins
30 of interest here are those which produce protein
complexes toxic to a variety of insects upon exposure, as
described herein. Preferably, the toxins are active
against *Lepidoptera*, *Coleoptera*, *Homoptera*, *Diptera*,
Hymenoptera, *Dictyoptera* and *Acarina*. The inventions
35 herein are intended to capture the protein toxins
homologous to protein toxins produced by the strains

herein and any derivative strains thereof, as well as any protein toxins produced by *Photorhabdus*. These homologous proteins may differ in sequence, but do not differ in function from those toxins described herein.

- 5 Homologous toxins are meant to include protein complexes of between 300 kDa to 2,000 kDa and are comprised of at least two 2) subunits, where a subunit is a peptide which may or may not be the same as the other subunit. Various protein subunits have been identified and are taught in the Examples herein. Typically, the protein subunits are between about 18 kDa to about 230 kDa; between about 160 kDa to about 230 kDa; 100 kDa to about 160 kDa; about 80 kDa to about 100 kDa; and about 50 kDa to about 80 kDa.

- As discussed above, some *Photorhabdus* strains can be isolated from nematodes. Some nematodes, elongated cylindrical parasitic worms of the phylum Nematoda, have evolved an ability to exploit insect larvae as a favored growth environment. The insect larvae provide a source of food for growing nematodes and an environment in which to reproduce. One dramatic effect that follows invasion of larvae by certain nematodes is larval death. Larval death results from the presence of, in certain nematodes, bacteria that produce an insecticidal toxin which arrests larval growth and inhibits feeding activity.

- Interestingly, it appears that each genus of insect parasitic nematode hosts a particular species of bacterium, uniquely adapted for symbiotic growth with that nematode. In the interim since this research was initiated, the name of the bacterial genus *Xenorhabdus* was reclassified into the genus *Photorhabditis* 30 *Photorhabdus*. Bacteria of the genus *Photorhabditis* characterized as being symbionts of *Heterorhabditis* nematodes while *Xenorhabdus* species are symbionts of the *Steinernema* species. This change in nomenclature is reflected in this specification, but in no way should a

change in nomenclature alter the scope of the inventions described herein.

The peptides and genes that are disclosed herein are named according to the guidelines recently published in
 5 the Journal of Bacteriology "Instructions to Authors" p. i-xii Jan. 1996), which is incorporated herein by reference.

Transformation methods useful in carrying out the invention are well known, and are described, for example,
 10 in W098/08932.

Hemicot tcdA and tcbA

SEQ ID NO: 3 is the nucleotide sequence for an engineered tcdA gene in accordance with the invention.
 SEQ ID NO: 4 is the nucleotide sequence for an engineered
 15 tcbA gene in accordance with the invention.

The following Tables 1 and 2 identify significant features of the engineered tcdA and tcbA genes.

Table 1
tcdA

Feature	nucleotides of SEQ ID NO:3
NcoI	1-6
HindIII	48-53
KpnI	246-254
sequence encoding TcbAii	267-5798
NheI	333-338
BglII	1215-1220
ClaI	2604-2609
PstI	4015-4020
AgeI	5088-5093
MunI	5598-5603
XbaI	5778-5783
sequence encoding TcbAiii	5799-7517
AflIII	5853-5858
SphI	6439-6444
SfuI	7392-7397
SacI	7519-7524
XhoI	7522-7527
StuI	7528-7533
NotI	7533-7538

20

Table 2
tcbA

Feature	nucleotides of SEQ ID NO:5
NcoI	1-6
HindIII	48-53

<i>KpnI</i>	246-251
sequence encoding <i>TcbAii</i>	267-5798
<i>NheI</i>	333-338
<i>BglII</i>	1215-1220
<i>ClaI</i>	2604-2609
<i>PstI</i>	4015-4020
<i>AgeI</i>	5088-5093
<i>MunI</i>	5598-5603
<i>XbaI</i>	5778-5783
sequence encoding <i>TcbAiii</i>	5799-7517
<i>AflIII</i>	5853-5858
<i>SphI</i>	6439-6444
<i>SfuI</i>	7392-7397
<i>SacI</i>	7519-7524
<i>SfuI</i>	7392-7397
<i>SacI</i>	7519-7524
<i>XhoI</i>	7522-7527
<i>StuI</i>	7528-7533
<i>NotI</i>	7535-7540

It should be noted that the proteins encoded by the plant-optimized *tcdA* (SEQ ID NO:3) and *tcbA* (SEQ ID NO:5) differ from the native proteins by the addition of an Ala residue at position #2. This modification was made to accommodate the *NcoI* site which spans the ATG start codon.

The following Table 3 compares the codon composition of the engineered *tcdA* gene of SEQ ID NO:3 and engineered *tcbA* gene of SEQ ID NO:5 with the codon compositions of the native genes, the typical dicot genes, and maize genes.

Table 3

amino acid	codon	% in SEQ ID NO:3	% in <i>tcdA</i>	% in SEQ ID NO:5	% in <i>tcbA</i>	% in dicot	% in maize
Ala	GCT	62	21	69	41	42	24
	GCC	26	32	27	17	27	34
	GCA	11	25	4	22	25	18
	GCG	0	21	0	21	6	24
Arg	AGG	48	0	60	2	25	26
	CGC	22	36	18	16	11	24
	AGA	20	11	15	6	30	15
	CGT	11	39	7	57	21	11
	CGG	0	7	0	13	4	15
	CGA	0	8	0	6	8	9
Asn	AAC	100	32	100	33	55	68
	AAT	0	68	0	67	45	32
Asp	GAC	67	22	70	25	42	63

amino acid	codon	% in SEQ ID NO:3	% in <i>tcdA</i>	% in SEQ ID NO:5	% in <i>tcbA</i>	% in <i>dicot</i>	% in <i>maize</i>
	GAT	33	78	30	75	58	37
Cys	TGC	100	30	100	19	56	68
	TGT	0	70	0	81	44	32
End	TGA	100	0	100	0	33	59
	TAG	0	0	0	0	19	21
	TAA	0	100	0	100	48	20
Gln	CAA	65	61	74	53	59	38
	CAG	35	39	26	47	41	62
Glu	GAG	100	24	98	36	51	71
	GAA	0	76	2	64	49	29
Gly	GGT	67	37	64	44	33	20
	GGC	32	36	36	22	16	42
	GGA	1	20	0	19	38	19
	GGG	0	8	0	16	12	20
His	CAC	62	40	72	31	46	62
	CAT	38	60	28	69	54	38
Ile	ATC	73	34	65	24	37	58
	ATT	27	51	35	59	45	28
	ATA	0	15	0	17	18	14
Leu	CTC	54	11	59	7	28	26
	TTG	29	17	25	32	26	15
	CTT	16	9	15	7	19	17
	TTA	0	18	0	19	10	5
	CTG	0	32	0	29	9	29
	CTA	0	13	0	7	8	8
Lys	AAG	99	79	99	75	61	78
	AAA	1	21	1	25	39	22
Met	ATG	100	100	100	100	100	100
Phe	TTC	100	42	100	41	55	71
	TTT	0	58	0	59	45	29
Pro	CCA	74	30	91	26	42	26
	CCT	22	28	7	20	32	22
	CCC	4	14	3	7	17	24
	CCG	0	27	0	47	9	28
Ser	TCC	47	19	55	11	18	23
	TCT	35	15	30	15	25	15
	AGC	18	22	15	18	18	23
	AGT	0	20	0	31	14	9
	TCG	0	7	0	8	6	14
	TCA	0	17	0	17	19	16
Thr	ACC	60	41	64	31	30	37
	ACT	28	25	32	34	35	20
	ACA	12	21	4	18	27	21
	ACG	0	13	0	18	8	22
Trp	TGG	100	100	100	100	100	100
Tyr	TAC	100	24	100	19	57	73
	TAT	0	76	0	81	43	27
Val	GTC	69	27	73	11	20	31
	GTG	21	17	22	27	29	39
	GTT	10	34	3	48	39	21
	GTA	0	22	2	14	12	8

EXAMPLE 1

Design Of Plant Codon-Biased Genes Encoding W-14 Peptides
TcbA and TcdA

5

A. Gene Design

The coding strands of the native DNA sequences of the *Photorhabdus* W-14 genes encoding peptides TcbA and TcdA were scanned for the presence of deleterious sequences such as the Shaw/Kamen RNA destabilizing motif ATTTA, intron splice recognition sites, and poly A addition motifs. This was done using the MacVector Sequence Analysis Software (Oxford Molecular Biology Group, Symantec Corp.), using a custom Nucleic Acid Subsequence File. The native sequence was also searched for runs of 4 or more of the same base.

Motif searching of the native W-14 *tcbA* and *tcdA* genes revealed the presence of many potentially deleterious sequences in the protein coding strands, as summarized in Table 4. Not shown, but also present, were many runs of four or more single residues (e.g. the native *tcbA* gene has 81 runs of four A's).

Table 4

Native Gene	ATTTA	5' Splice	3' Splice	Poly A Addition*	RNAP II term.
<i>tcbA</i>	18	7	17	46	0
<i>tcdA</i>	18	7	13	77	1

* Totals of 16 different motifs.

Analyses of eukaryotic genes and plant genes in particular have shown that CG & TA doublets are underrepresented, while the genes are enriched in CT & TG doublets. The sequences of the hemicot biased genes have accordingly been adjusted to encompass these base compositions and to have G+C compositions of about 53%, similar to many plant genes. When compared to the native W-14 *tcbA* and *tcdA* genes, the plant-biased genes have a much more uniform G+C distribution.

Nucleotide changes to remove potentially deleterious sequences were chosen to simultaneously adjust the codon composition of the coding region to more closely reflect that of plant genes. A framework for these changes was provided by the codon bias tables prepared for maize and dicot genes shown in Table 3.

Comparison of codon compositions of the native W-14 genes to maize and dicot genes revealed that the W-14 genes contain a very different preference set of the degenerate codons for the 18 amino acids for which there is a choice (Table 3). For each of 8 amino acids (Phe, Tyr, Cys, Arg, Asn, Lys, Glu, and Gly) in both W-14 genes, the most abundant codon is different from the preferred codons found in either maize or dicot genes. One might expect that translational difficulties would be encountered in efforts to produce in plants proteins (such as TcbA and TcdA) having high relative amounts of these amino acids from mRNAs having large numbers of nonpreferred codons. There is a marked difference in distribution of the codon compositions specifying the other 10 amino acids. For His, Gln, Ile, Val, and Asp, the dicot-preferred codons are found as the most abundant ones in both W-14 genes. For Leu, Thr, Ser, and Ala, the maize preferred codons are the most abundant codon choices found in the *tcdA* gene. In contrast, the *tcbA* gene contains only the CCG (Pro) maize-preferred codon as the highest abundance choice.

In making the codon choices, doublet contents were considered, so that adjacent codons preferably did not form CG or TA doublets (which are underrepresented in eukaryotic genes; 1, 4), while CT or TG doublets (which are enriched in eukaryotic genes ibid.) were created when possible.

Choices were also made to utilize a diversity of codons for Met, Trp, Asn, Asp, Cys, Glu, His, Ile, Lys, Phe, Thr, and Tyr.

The sequences were also designed to encode unique 6-bp recognition sites for restriction enzymes, spaced about every 1200 bp. Finally, an additional codon (GCT; Ala) was inserted at the second position to encode an Nco I recognition site encompassing the ATG (Met) start codon. Additional recognition sites were included after

the stop codon to facilitate subsequent cloning steps into expression vectors. These features are set forth above in Tables 1 and 2.

The new *tcdA* and *tcbA* genes of SEQ ID NO:3 and SEQ ID NO:4 share 73.5%, and 72.6% identity, respectively, to their native W-14 counterparts (Wisconsin Genetics Computer Group, GAP algorithm).

B. Gene Synthesis

The complete synthesis of the plant codon-biased *tcbA* and *tcdA* genes was performed under contract by Operon Technologies, Inc. (OPTI, Alameda, CA). Basically, chemically synthesized oligonucleotides of appropriate sequence were assembled into DNA pieces about 500 bases long. These were joined together end-to-end (presumably by means of appropriately placed restriction enzyme sites) into four larger pieces of roughly 2 kilobase pairs (kbp) each; therefore each comprised about 1/4 of the entire coding region of the particular gene. DNA sequence of the pieces was confirmed at this step. If mistakes in sequence were present, the appropriate oligonucleotides were re-synthesized, and the assembly process was repeated. Once gene fractional parts were sequence verified, they were assembled in pairs to make the gene halves, and again sequence verified. Finally, the two halves were joined, and the sequences of the junctions between the halves was verified. Therefore, each part of the new gene was sequence verified at least twice.

It should be noted that attempts to express the native *tcbA* or *tcdA* genes in standard *Escherichia coli* cloning strains suggests that production of these proteins is lethal. Lethality problems may be encountered if standard cloning vectors having leaky expression from inherent *lacZ* promoters are used to assemble these genes.

C. Addition Of Endoplasmic Reticulum Targeting Peptide To Tcda Coding Region

It is known to those in the field of plant gene expression that proteins are specifically directed into the endoplasmic reticulum (ER) by means of a short signal peptide which is removed during or after the transport process through the ER membrane. The mature (processed) protein is incorporated into the ER endomembrane or is released into the ER lumen where the transported protein may be uniquely folded (aided by chaperonins), modified by glycosylation, accumulated in the vacuole, or additionally translocated (by secretion). These processes are reviewed by Gomord and Faye [V. Gomord and L. Faye, (1996) *Signals and mechanisms involved in intracellular transport of secreted proteins in plants*. Plant Physiol. Biochem. 34:165-181] and by Bar-Peled et al. [M. Bar-Peled, D. C. Bassham, and N. V. Raikhel, (1996) *Transport of proteins in eukaryotic cells: more questions ahead*. Plant Molec. Biology 32:223-249]. It is also known that the subcellular recognition mechanisms for an ER signal peptide are evolutionarily somewhat conserved, since the ER signal for a protein normally produced in monocot (maize) cells is recognized and processed normally by dicot (tobacco) cells. This is exemplified by the maize 15 kDa zein ER signal peptide [L. M. Hoffman, D. D. Donaldson, R. Bookland, K. Rashka, and E. M. Herman, (1987) *Synthesis and protein body deposition of maize 15-kd zein in transgenic tobacco seeds*. EMBO J. 6:3213-3221, and U.S. Patent 5589616]. Further, it is known that the ER signal peptide derived from one protein can direct the translocation of a different protein if it is appropriately attached to the second protein by genetic engineering methods [D. C. Hunt and M. J. Chrispeels, (1991) *The signal peptide of a vacuolar protein is necessary and sufficient for the efficient secretion of a cytosolic protein*. Plant

Physiol. 96:18-25, and Denecke, J., J. Botterman, and R. Deblaere (1990) *Protein secretion in plants can occur via a default pathway*. Plant Cell 2:51-59]. Therefore, one may expose a protein in vivo to different biochemical environments by directing its accumulation in the cytosol (by not providing a signal peptide sequence), or in the ER/vacuole (by provision of an appropriate signal peptide.)

The ER signal peptide of maize 15 kDa zein proteins is known to comprise the first 20 amino acids encoded by the zein coding region. Two examples of such signal peptides the ER signal peptide of 15 kDa zein from A5707 maize, NCBI Accession # M72708, and the ER signal peptide of 15 kDa zein from Black Mexican Sweet maize, NCBI Accession # M13507. There is only a single amino acid difference (Ser vs Cys at residue 17) between these signal peptides.

SEQ ID NO:5 is a modified sequence coding the ER signal peptide of 15 kDa zein from Black Mexican Sweet maize. The modifications embodied in this sequence were made to accommodate the different monocot/dicot codon usages and other sequence motif considerations discussed above in the design of the plant-optimized *tcdA* coding region. The sequence includes an additional Ala residue at position #2 to accommodate the *NcoI* site which spans the ATG start codon.

SEQ ID NO:6 gives a sequence coding for the full-length native TcdA protein (amino acids 22-2537) fused to the modified 15 kDa zein endoplasmic reticulum signal peptide (amino acids 1-21).

Example 2

Transformation Of Tobacco With *Agrobacterium* Carrying Plasmid pDAB2041 Encoding *Photobacterium* Toxins
A. Plasmid pDAB2041

Preparation of tobacco transformation vectors was accomplished in three steps. First, a modified plant-optimized *tcdA* coding region was ligated into a tobacco

plant expression cassette plasmid. In this step, the coding region was placed under the transcriptional control of a promoter functional in tobacco plant cells. RNA transcription termination and polyadenylation were mediated by a downstream copy of the terminator region from the *Agrobacterium* nopaline synthase gene. Two plasmids designed to function in this role are pDAB1507 and pDAB2006. In the second step, the complete gene comprised of the promoter, coding region, and terminator region was ligated between the T-DNA borders of an *Agrobacterium* binary vector, pDAB1542. Also positioned between the T-DNA borders was a plant selectable marker gene to allow selection of transformed tobacco plant cells. In the third step, the engineered binary vector plasmid was conjugated from its *E. coli* host strain into a disabled *Agrobacterium tumefaciens* strain capable of transforming tobacco plant cells that regenerate into fertile transgenic plants.

It is a feature of plasmid pDAB1507 that any coding region having an *Nco*I site at its 5' end and a *Sac*I site 3' to the coding region, when cloned into the unique *Nco*I and *Sac*I sites of pDAB1507, is placed under the transcriptional control of an enhanced version of the CaMV 35S promoter. It is also a feature of pDAB1507 that the 5' untranslated leader (UTR) sequence preceding the *Nco*I site comprises a modified version of the 5' UTR of the MSV coat protein gene, into which has been cloned an internally deleted version of the maize *Adh1S* intron 1. Additionally it is a feature of pDAB1507 that transcription termination and polyadenylation of the mRNA containing the introduced coding region are mediated by termination/Poly A addition sequences derived from the nopaline synthase (Nos) gene. Finally, it is a feature of pDAB1507 that the entire assembly of promoter/coding region/3'UTR can be obtained as a single DNA fragment by cleavage at the flanking *Not*I sites.

It is a feature of plasmid pDAB2006 that any coding region having an *Nco*I site at its 5' end and a *Sac*I site 3' to the coding region, when cloned into the unique *Nco*I and *Sac*I sites of pDAB2006, is placed under the transcriptional control of the CaMV 35S promoter. It is also a feature of pDAB2006 that the 5' untranslated leader (UTR) sequence preceding the *Nco*I site comprises a polylinker. Additionally it is a feature of pDAB2006 that transcription termination and polyadenylation of the mRNA containing the introduced coding region are mediated by termination/Poly A addition sequences derived from the nopaline synthase (*Nos*) gene. Finally, it is a feature of pDAB2006 that the entire assembly of promoter/coding region/3'UTR can be obtained as a single DNA fragment by cleavage at the flanking *Not*I sites.

It is a feature of pDAB1542 that any DNA fragment flanked by *Not*I sites can be cloned into the unique *Not*I site of pDAB1542, thus placing the introduced fragment between the T-DNA borders, and adjacent to the neomycin phosphotransferase II (kanamycin resistance) gene.

To prepare a plant-expressible gene to produce the non-targeted TcdA protein in tobacco plant cells, DNA of a plasmid (pAOH_4-OPTI) containing the plant-optimized *tcdA* coding region, (SEQ ID No:3) was cleaved with restriction enzymes *Nco*I and *Sac*I, and the large 7550 bp fragment was ligated to similarly-cut DNA of plasmid pDAB1507 to produce plasmid pDAB2040. DNA of pDAB2040 was then digested with *Not*I, and the 8884 bp fragment was ligated to *Not*I digested DNA of pDAB1542 to produce plasmid pDAB2041. This plasmid was then conjugated by triparental mating [Firoozabady, E., D. L. DeBoer, D. J. Merlo, E. L. Halk, L. N. Amerson, K. E. Rashka, and E. E. Murray (1987) *Transformation of cotton (Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants. *Plant Molec. Biol.*

10:105-116] from the host *Escherichia coli* strain (XL1-Blue, Stratagene, La Jolla, CA), into the nontumorigenic *Agrobacterium tumefaciens* strain EHA101S, which is a spontaneous streptomycin-resistant mutant of strain
5 EHA101 (Hood, E. E., G. L. Helmer, R. T. Fraley, and M.-D. Chilton (1986) *The hypervirulence of Agrobacterium tumefaciens A281 is encoded in a region of pTiBo542 outside of T-DNA*. J. Bacteriol. 168:1291-1301). Strain EHA101S(pDAB2041) was then used to produce transgenic
10 tobacco plants that expressed the TcdA protein.

B. Plasmid pRK2013

To prepare a plant-expressible gene to produce the endoplasmic reticulum-targeted TcdA protein in tobacco plant cells, DNA of a plasmid (pA0H_4-ER) containing the
15 plant-optimized, ER-targeted *tcdA* coding region, (SEQ ID No:6) was cleaved with restriction enzymes *NcoI* and *SacI*, and the large 7610 bp fragment was ligated to similarly-cut DNA of plasmid pDAB2006 to produce plasmid pDAB1833. DNA of pDAB1833 was then digested with *NotI*, and the 8822
20 bp fragment was ligated to *NotI* digested DNA of pDAB1542 to produce plasmid pDAB2052. This plasmid was then conjugated by triparental mating from the host *Escherichia coli* strain (XL1-Blue), into the nontumorigenic *Agrobacterium tumefaciens* strain EHA101S.
25 Strain EHA101S(pDAB2052) was then used to produce transgenic tobacco plants that expressed the TcdA protein containing an amino terminus endoplasmic reticulum targeting peptide.

30 C. Transfer of Plasmid pDAB2041 Into *Agrobacterium* Strain EHA101S

Cultures of *E. coli* carrying the engineered Ti plasmid pDAB2041 (plasmid containing the rebuilt Toxin A gene, *tcdA*), *E. coli* carrying the plasmid pRK2013, and
35 *Agrobacterium* strain EHA101S were grown overnight, then mixed 1:1:1 on plain LB medium solidified with agar and

cultured in the dark at 28°C. Two days later, the lawn of bacteria was scraped up with a loop, suspended in plain LB medium, vortexed, and then diluted 1:10⁴, 1:10⁵, and 1:10⁶ fold in plain LB liquid medium. Aliquots of these dilutions were spread on selective plates containing medium YEP plus erythromycin (100 mg/L) and streptomycin (250 mg/L) and grown at 28°C. Two days later, single colonies were picked and streaked onto the same medium, then spread to give single colonies. Single colonies were picked again and streaked, then spread for single colonies. Single colonies were picked a third time, grown as streaks, then subjected to a quality analysis involving growth on lactose medium and chromogenic assay with Benedict's reagent. Of ten strains developed in this way, the fastest coloring colony was chosen for further work.

D. Transformation Of Tobacco With *Agrobacterium* Carrying Plasmid pDAB2041

Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (R Horsch et al, 1988. Plant Molecular Biology Manual, S. Gelvin et al, eds., Kluwer Academic Publishers, Boston). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. Kentucky 160) were surface sterilized and planted on the surface of TOB-, which is a hormone-free Murashige and Skoog medium (T. Murashige and F. Skoog, 1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Plant Physiol. 75: 473-497) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30°C and leaves were collected sterilely for use in the transformation protocol. Approximately one cm² pieces were sterilely cut from these leaves, excluding the midrib. Cultures of the

Agrobacterium strains (EHA101S containing pDAB2041), which had been grown overnight on a rotor at 28°C, were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, adjusted to a final optical density of 0.7 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on medium TOB+ (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28°C. Two days later the leaf pieces were moved to medium TOB+ containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Florida) and 100 mg/L kanamycin sulfate (AgriBio) and incubated at 28-30°C in the light. Leaf pieces were moved to fresh TOB+ with cefotaxime and kanamycin twice per week for the first two weeks and once per week thereafter. Leaf pieces which showed regrowth of the *Agrobacterium* strain were moved to medium TOB+ with cefotaxime and kanamycin, plus 100 mg/l carbenicillin (Sigma). Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB- containing 250 mg/L cefotaxime and 100 mg/L kanamycin in Magenta GA7 boxes (Magenta Corp., Chicago). These plantlets were grown in a lighted incubator room. After 3-4 weeks the primary transgenic plants had rooted and grown to a size sufficient that leaf samples could be analyzed for expression of protein from the transgene. Twenty-five independent transgenic events were recovered as single plants from the pDAB2041 transformation.

Eight independent lines expressing various levels of transgenic protein from the T-DNA of pDAB2041 were propagated *in vitro* from leaf pieces as follows. Twelve to sixteen approximately one cm² pieces were sterily cut from leaves of each primary transgenic plant, excluding

the midrib and all naturally occurring edges. These leaf pieces were placed on medium TOB+ containing 250 mg/L cefotaxime and 100 mg/L kanamycin, and cultured in the lighted incubator at 28-30°C for 3-4 weeks, at which time
5 small plants could be cut from the proliferating tissue mass. Several small plantlets from each transgenic line were moved into Magenta boxes containing medium TOB- plus cefotaxime and kanamycin and allowed to root and grow. The proliferating tissue mass was further cultured on
10 medium TOB+ with cefotaxime and kanamycin, and additional plants could be cut out and grown up as needed.

Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 5 1/4" square pots, placing the pot into a Ziploc bag
15 (DowBrands), placing plain water into the bottom of the bag, and placing in indirect light in a 30°C greenhouse for one week. After one week the bag could be opened; the plants were fertilized and allowed to grow further, until the plants were acclimated and the bag was removed.
20 Plants were grown under ordinary warm greenhouse conditions (30°C, 16 H light). Plants were suitable for sampling four weeks post transplant.

Example 3

25 Characterization Of Transgenic Tobacco Plants Expressing Photorhabdus Toxin That Confer Insect Control.

A. Polyclonal Antibody Production

The *E. coli* produced recombinant TcdA protein was
30 purified by a series of column purification. The protein was sent to Berkley Antibody Company (Richmond, CA) for the production of antiserum in a rabbit. Inoculations with the antigen were initiated with 0.5 mg of protein followed by four boosting injections of 0.25 mg each at
35 about three week intervals. The rabbit serum was tested by the standard Western analysis using the recombinant TcdA protein as the antigen and enhanced chemi-

luminescens, ECL method (Amersham, Arlington Heights, IL) .The antibodies (PAb-EA₀) were purified using a PURE I antibody purification kit (Sigma, St. Luis, MO). PAb-EA₀ antibodies recognize the full-length TcdA and its
5 processed components.

B. Expression Of TcdA Protein In Tobacco

Protein was extracted from the leaf tissue of transformed and non-transformed tobacco plants following the procedure described immediately below.

10 Two leaf disks of 1.4 cm in diameter were harvested from the middle portion of a fully expanded leaf. The disks were placed on a 1.6 x 4 cm piece of 3M Whatman paper. The paper was folded lengthwise and inserted in a flexible straw. Four hundred micro liters of the
15 extraction buffer (9.5 ml of 0.2 M NaH₂PO₄, 15.5 ml of 0.2 M Na₂HPO₄, 2 ml of 0.5 M Na₂EDTA, 100 ml of Triton X100, 1 ml of 10% Sarkosyl, 78 ml of beta-mercaptoethanol, H₂O to bring total volume to 100 ml) was pipetted on to the paper. The straw containing the sample was then passed
20 through a rolling device used for squeezing out the extract 1.5 mL micro centrifuge tube was placed at the other end of the straw to collect the extract. The extract was centrifuged for 10 minutes at 14,000 rpm in an Eppendorf regrigerated microcentrifuge. The
25 supernatant was transferred into a new tube. Protein quantitation analysis was performed using the standard Bio-Rad Protein Analysis protocol (Bio-Rad Laboratories, Hercules, CA). The extract was diluted to 2 mg/ml of total protein using the extraction buffer.

30 For the detection of transgenic protein, Western blot analysis was performed. Following a standard procedure for protein separation (Laemmli, 1970), 40 µg of protein was loaded in each well of 4-20% gradient polyacrylamide gel (Owl Scientific Co., MA) for
35 electrophoresis. Subsequently, the protein was

transferred onto a nitrocellulose membrane using a semi-dry electroblotter (Pharmacia LKB Biotechnology, Piscataway, NJ). The membrane was incubated for one hour in Blotto (5% milk in TBST solution; 25 mM Tris HCL pH 7.4, 136 mM NaCl, 2.7 mM KCl, 0.1% Tween 20). Thereafter, Blotto was replaced by the primary antibody solution (in Blotto). After one hour in the primary antibody, the membrane was washed with TBST for five minutes three times. Then the secondary antibody in Blotto (1:2000 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase; Bio-Rad Laboratories). was added to the membrane. After one hour of incubation, the membrane was washed with an excess amount of TBST for 10 minutes four times. The protein was visualized by using the enhanced chemi-luminescens, ECL method (Amersham, Arlington Heights, IL). The differential intensity of the protein bands were measured using densitometer (Molecular Dynamics Inc., Sunnyvale, CA).

To determine the expression of TcdA protein in tobacco transformed with pDAB2041, PAb-EA₀ antibodies were used as the primary antibodies. The expression levels of TcdA protein varied among independent transformation events. The primary plant generated from the event #2041-13 showed the highest level of pre-pro TcdA expression of extractable protein. When the leaf pieces from this plant (#2041-13) were used in *in vitro* propagation, several plants were obtained. Seven of these plants were analyzed for the expression of the TcdA protein. All but one plant produced the full-length TcdA protein as well as some processed peptide components. Using the antibodies specific to Neomycin phosphotransferase, NPT (5 prime-3 prime, Boulder, Co), the expression the selectable marker gene (*npt II*) was detected. Similar results were obtained for #2041-29.

35

Table 5

Western analysis of plants derived from event #2041-13.

Plant #	TcdA	NPT (selectable marker)
2041-13A	+	not done
2041-13B	+	not done
2041-13-1	-	+
2041-13-2	+	+
2041-13-3	+	+
2041-13-4	+	+
2041-13-5	+	+

C. Nucleic Acid Analysis of Transgenic Tobacco Lines

Genomic DNA was prepared from a group of 2041
 5 transgenic events. The lines included Magenta box stage
 2041-13, and greenhouse stage plants 2041-13-1, 2041-13-
 2, 2041-13-5, 2041-9, 2041-20A and 2041-20B. A
 transgenic GUS line (2023) was included as a negative
 control. Southern analysis of these lines was performed.
 10 The genomic tobacco DNA was restricted with the enzyme
 SstI which should result in a 8.9 kb hybridization
 product when hybridized to a *tcdA* gene specific probe.
 The 8.9 kb hybridization product should consist of the
 35T promoter and the *tcdA* coding region. All 2041 plants
 15 contained a band of the expected size. Events 2041-9 and
 -20 appear to be the same line with 5 identical
 hybridizing bands. Event 2041-13 produced 6
 hybridization fragments with the *tcdA* coding region
 probe. Magenta box and various greenhouse plants of
 20 2041-13 all produced the same hybridization profile.
 This hybridization pattern was different from that of
 events 2041-9 and -20.

RNA analysis, using the *tcdA* coding region probe,
 was performed on the same group of greenhouse 2041
 25 plants. Immunoblot analysis had revealed that plants
 2041-9, 2041-20A, 2041-20B, and 2041-13-1 produced no
 detectable TcdA protein; while 2041-13-2 and 2041-13-5
 produced substantial amounts of full-length TcdA.
 Northern analysis was in agreement with the immunoblot

result. A faint RNA signal was detected for plants 2041-9, 2041-20A, 2041-20B, and 2041-13-1. Only faintly visible was a band corresponding to full-length *tcdA* transcript in plant 2041-13.1. In contrast, for plants 5 2041-13-2 and 2041-13-5 a strong RNA signal was detected, with a substantial amount of full-length size (~8.0 kb) *tcdA* transcript. These data support the observed bioassay activity for this group of plants.

Genomic DNA was prepared from a second functionally 10 active 2041 transgenic event, 2041-29. Southern analysis of this line was performed. A transgenic GUS line (2023) was included as a negative control, DNA of line 2041-9 was included as a positive control.

The genomic tobacco DNAs were restricted with the 15 enzyme *Sst*I which should result in a 8.9 kb hybridization product when hybridized to a *tcdA* gene specific probe. The 8.9 kb hybridization product should consist of the 35T promoter and the *tcdA* coding region. For plant 2041-29-5, three hybridization products larger than 8.9 kb the 20 were detected with the *tcdA* gene specific probe. Immunoblot analysis has demonstrated pre-pro TcdA protein is made by this plant, it is therefore likely that a restriction site was lost during transformation or regeneration, or the 2041-29 genomic DNA was not 25 thoroughly digested.

D. Tobacco Leaf-Disk Tests With Tobacco Hornworm Exhibiting Insect Control

Leaves were sampled from tobacco plants, *Nicotiana* 30 *tabaco*, previously transplanted into the greenhouse. A single leaf was sampled from each plant on each test date. Leaves were selected from the zone where younger elongate leaves transition into older ovate leaves. Excised leaves were placed into 12 oz. cups with the 35 petiole submerged in water to maintain turgor, and transported to the laboratory.

Eight, 1.4 cm disks were cut from the center portion of one side of each leaf (right adaxial side up, with distal portion facing away from the observer). Each disk was placed individually into a well of a C-D

5 International 128 well tray (Pitman, NJ.) into which 0.5 ml of a 1.6% aqueous agar solution had been previously pipetted. The solidified agar prevented the leaf disks from drying out. The adaxial surface of the disk was always oriented up.

10 A single neonate tobacco hornworm, *Manduca sexta*, was placed on each disk and the wells were sealed with vented plastic lids. The assay was held at 27°C and 40% RH. Larval mortality and live-weight data were collected after 3 days. Data were subjected to analysis of
15 variance and Duncan's multiple range test ($\alpha = 0.05$) (Proc GLM, SAS Institute Inc., Cary, NC.). Data were transformed using a logarithmic function to correct a correlation between the magnitude of the mean and variance.

20 Table 6
Results of leaf-disk assays from greenhouse grown tobacco plants with event 2041-13.

TRT	Plant	Plant Age	Weight of Surviving Larvae (mg) & Duncan's Group ¹				
			Pretes t	Test 1	Test 2	Test 3	3 Test Sum.
13	non-transformed - 2	young	---	---	---	18.8 a*	---
14	non-transformed - 3	young	---	---	---	17.0 ab	---
16	non-transformed - 5	young	---	---	---	16.4 ab	---
3	2041-13-1 (western -)	young	---	17.6 a	18.2 a	16.1 ab	17.3 a
9	Gus Control	old	19.3 a	14.6 a	16.3 a	14.5 ab	15.1 a
10	non-transformed - 1	young	---	8.3 b	16.8 a	13.9 b	13.0 b
11	2041-20B (western -)	old	---	10.0 b*	13.7 ab	14.6 ab	12.9 b
15	non-transformed - 4	young	---	---	---	13.0 bc	---
8	2041-20A (western -)	old	15.7 a	8.3 b	11.3 bc	9.2 cd	9.6 c
12	2041-9 (western -)	old	19.5 a	---	---	7.9 d	---
7	2041-13-5 (western +)	young	---	6.3 bc	9.6 cd	7.2 de	7.7 d
5	2041-13-3 (western +)	young	---	6.4 bc****	6.2 c	6.8 de**	6.4 de
1	2041-13A (western +)	old	7.2 b	6.8 bc*	7.0 de*	5.4 e	6.4 de
6	2041-13-4 (western +)	young	---	4.9 c****	5.8 e	7.6 d	6.4 de
4	2041-13-2 (western +)	young	---	5.7 bc	5.7 e**	7.5 d	6.3 de
2	2041-13B (western +)	old	---	4.7 c**	5.6 e	7.2 de	5.9 e

* Number of stars corresponds to the number of dead larvae per 8 tested.

1. Data transformed (logarithm) for analysis.

Means followed by the same letter are not significantly different ($\alpha = 0.05$).

5

TABLE 7
Results Of Leaf-Disk Assays From Greenhouse Grown Tobacco
Plants
With Event 2041-29.

Plant	MEAN WGT (MG) / Duncan's Group				
	Test 1	Test 2	Test 3	Test 4	Four Test Summary
2014-6 GUS 1	15.8 a	16.6a	**5.5bc	*12.9ab	13.2 a
2014-6 GUS 2	14.4 a	*6.6 bc	*13.4a	15.2a	12.6 a
KY-160 NTC	13.4 a	6.7 bc	7.9b	8.5bc	9.1 b
2041-29 4P	*4.9 b	*7.3b	****6.9b	*****	6.3 c
2041-29 7	*5.9 b	5.1bc	***6.7b	***7.2c	6.1 c
2041-29 3P	*5.6 b	**7.9b	*****6.5b	***3.6d	5.9 c
2041-29 2P	6.3 b	****4.7c	*****4.1c	*****4.6d	5.4 c

* Number of stars corresponds to the number of dead larvae per 8 tested.

10

1. Data transformed (logarithm) for analysis.

Means followed by the same letter are not significantly different ($\alpha = 0.05$).

All event 2041-29 plants significantly depressed THW

15

larval weight gain compared to control plants. Average weight depression was 49%. Statistically significant mortality occurred in THW larvae exposed to foliage from 2041-29 plants. Mortality averaged 37.5% compared to 5.2% in controls.

20

E. Isolation and Characterization of Functional Photorhabdus Toxin Protein From Transgenic Plants

Seven grams of transgenic tobacco plants (2041-13) expressing TcdA (Toxin A) gene were homogenized with 10 ml 50 mM Potassium Phosphate buffer, pH 7.0 using a bead beater (Biospec Products, Bartlesville, OK) according to manufacturer's instructions. The homogenate was filtered through four layers of cheese cloth and then centrifuged at 35,000 g for 15 min. The supernant was collected and filtered through 0.22 μ m Millipore Express™ membrane. It was then applied to a Superdex 200 cloumn (2.6 x 40 cm)

which had been equilibrated with 20 mM Tris buffer, pH 8.0 (Buffer A). The protein was eluted in Buffer A at a flow rate of 3 ml/min. Fractions with 3 ml each were collected and subjected to southern corn rootworm (SCR) bioassay. It was found that fractions corresponding to a native molecular weight around 860 kDa had the highest insecticidal activity. Western analysis of the active fraction using a polyclonal antibody specific to Toxin A indicated the presence of full-length TcdA peptide. The active fractions were further combined and applied to a Mono Q 10/10 column which had been equilibrated with Buffer A. Proteins bound to the column were then eluted by a linear gradient of 0 to 1 M NaCl in Buffer A. Fractions with 2 ml each were collected and analyzed by both SCR bioassay and Western using antibody specific to Toxin A. The results again demonstrated the correlation between insecticidal activity and presence of full-length TcdA peptide.

20 F. Characterization of Progeny Transgenic Plants

The inheritability of the genetically engineering plants containing the *Photorhabdus* toxin gene was evaluated by generating F1 progeny. Progeny was generated from 2041-13 event by selfing expression positive plants. The 2041-13 plants in the greenhouse were allowed to self-pollinate. Seed capsules were collected when mature and were allowed to dry and after-ripen on the laboratory bench for two weeks. Seed from plant designated 2041-13A was surface-sterilized and distributed on the surface of medium TOB- without selection, to allow recovery of nonexpressing or nontransgenic progeny as well as expressing and segregating transgenic siblings. Seed was germinated in a C lighted incubator room (16 H light, 28 C). After 1 month, fifty-one seedlings, designated 2041-13A-S1 through S51, were distributed into Magenta boxes

containing medium TOB- to grow further. Three weeks later, leaf samples from these Magenta-box grown seedlings were submitted for evaluation of the level of expression of TcdA toxin.

5 Leaf samples were tested for kanamycin response by placing sterile leaf segments on medium TOB+ containing 100 mg/L kanamycin in the light and scoring for tissue growth and color after two weeks. All leaf pieces showed some positive response, indicating complex segregation.

10 This group of in vitro grown event 2041-13 progeny seedlings were all transplanted into the greenhouse approximately two months after seeding onto medium, using the following method. After washing the agar from the roots, plants were transplanted into 5 ½ inch square pots
15 in a soil mix containing 75% MetroMix and 25% mineral soil. They were enclosed in a zip-lock bag and plain water added to leave 1-2 inches of water in the bottom of the bag after soil absorption. These bags were closed and placed under a cart in the greenhouse to protect them
20 from direct sunlight. The bags were opened after 5-6 days, and removed after 7 days, when the plants were adapted to soil and were moved to the top of the cart for normal greenhouse culture. Plants were ready to test in insect bioassays at four weeks post transplant.

25 F1 progeny were evaluated for expression of protein toxin by immunological screen and for biological activity by plant bioassays, as described previously, using tobacco hornworm. There existed a positive correlation between levels of expression protein toxin and degree of
30 growth inhibition and at higher expression levels mortality was observed. The biological activity was observed to be statistical significance with high confidence levels between populations of non-transformed and transformed expressing protein toxin.

35 The following table summarizes the results of insect (tobacco hornworm) bioassays conducted with F1 progeny of

self-fertilized 2041-13 plants genetically engineered to produce the "204" A toxin. The tests included 6 non-expressing progeny (protein-negative controls), 45 toxin A expressors, and 4 non-transformed controls (KY-160).

- 5 Results are from three leaf-disk assays (method previously outlined) where eight disks were used per test. The data were analyzed using analysis of variance and were blocked by test.

The treatment effect for each of these analyses indicated the $Pr > F$ was less than 0.0001. The Toxin A expressors produced significant control of tobacco hornworm compared to each of the control groups based on each of the three measures of efficacy. The two control groups behaved similarly. Statistical analysis using ANOVA and an LSD test with alpha equal to 0.01 (or 1%) showed differences between the 3 groups. The LSD test indicated that the non-expressors and the non-transformed plants were similar in larvae weights but the expressors gave weights significantly lower than either of the other two groups of plants. These data demonstrated that the genetic basis for insect control was inheritable and corresponded to the presence of expressed toxin gene.

Table 8
Tobacco hornworm results from F1 progeny of self-fertilized
2041-13 tobacco plants.

Treatment Group	Mean Value and Duncan's Grouping ^d		
	Total Weight (mg) ^a	Survivor Weight (mg) ^b	Leaf Area (cm ²) ^c
Non-transformed Control	15.8 a	15.8 a	1.2 a
Protein-negative Control	16.4 a	16.5 a	1.2 a
Toxin A Expressor	8.1 b	9.2 b	4.9 b

^a Average insect weight with dead insects considered to weigh nothing.

^b Average insect weight with dead insects excluded from analysis.

^c Total leaf area remaining per eight leaf disks. Initial area was approximately 12 cm².

^d Means followed by the same letter are not significantly different ($\alpha = 0.05$).

Example 4

5 Transformation Of Maize With a Vector Carrying Plasmid
pDAB1834 Encoding *Photorhabdus* Toxins

A. Preparation Of Maize Transformation Vectors
Containing Modified Plant-Optimized *TcdA* Coding Regions:
Plasmid Pdab1834

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Preparation of maize transformation vectors was
accomplished in two steps. First, a modified plant-
optimized *tcdA* coding region was ligated into a plant
expression cassette plasmid. In this step, the coding
15 region was placed under the transcriptional control of a
promoter functional in maize plant cells. RNA
transcription termination and polyadenylation were
mediated by a downstream copy of the terminator region
from the *Agrobacterium nopaline* synthase gene. One
20 plasmid designed to function in this role is pDAB1538. In
the second step, the complete gene comprised of the
promoter, coding region, and 3' UTR terminator region was
ligated to a plant transformation vector that contained a
plant expressible selectable marker gene which allowed
25 the selection of transformed maize plant cells amongst a
background of nontransformed cells. An example of such a
vector is pDAB367.

It is a feature of plasmid pDAB1538 that any coding
region having an *NcoI* site at its 5' end and a *SacI* site
30 3' to the coding region, when cloned into the unique *NcoI*
and *SacI* sites of pDAB1538, is placed under the
transcriptional control of the maize ubiquitin1 (*ubi1*)
promoter. It is also a feature of pDAB1538 that the 5'
untranslated leader (UTR) sequence preceding the *NcoI*
35 site comprises a polylinker. Additionally it is a
feature of pDAB1538 that transcription termination and
polyadenylation of the mRNA containing the introduced
coding region are mediated by termination/Poly A addition

sequences derived from the nopaline synthase (Nos) gene. Finally; it is a feature of pDAB1538 that the entire assembly of promoter/coding region/3'UTR can be obtained as a single DNA fragment by cleavage at the flanking NotI sites.

It is a feature of pDAB367 that the phosphinothricin acetyl transferase protein, which has as its substrate phosphinothricin and related compounds, is produced in plant cells through transcription of its coding region mediated by the Cauliflower Mosaic Virus 35S promoter and that termination of transcription plus polyadenylation are mediated by the nopaline synthase terminator region. It is further a feature of pDAB367 that any DNA fragment containing flanking NotI sites can be cloned into the unique NotI site of pDAB367, thus physically linking the introduced DNA fragment to the aforementioned selectable marker gene.

To prepare a maize plant-expressible gene to produce the endoplasmic reticulum-targeted TcdA protein in plant cells, DNA of a plasmid (pAOH_4-ER) containing the plant-optimized, ER-targeted *tcdA* coding region, (SEQ ID No:6) was cleaved with restriction enzymes *NcoI* and *SacI*, and the large 7610 bp fragment was ligated to similarly-cut DNA of plasmid pDAB1538 to produce plasmid pDAB1832. DNA of pDAB1832 was then digested with *NotI*, and the 9984 bp *NotI* fragment was ligated into the unique *NotI* site of pDAB367 to produce plasmid pDAB1834.

It is a feature of plasmids pDAB1834 that the *ubil* and 35S promoters are encoded on the same DNA strand.

30

B. Transformation and Regeneration of Transgenic Maize Isolates

Type II callus cultures were initiated from immature zygotic embryos of the genotype "Hi-II." (Armstrong et al, (1991) Maize Genet. Coop. Newslett., 65: 92-93). Embryos were isolated from greenhouse-grown ears from

35

crosses between Hi-II parent A and Hi-II parent B or F₂ embryos derived from a self- or sib-pollination of a Hi-II plant. Immature embryos (1.5 to 3.5 mm) were cultured on initiation medium consisting of N6 salts and vitamins (Chu et al, (1978) *The N6 medium and its application to anther culture of cereal crops*. Proc. Symp. Plant Tissue Culture, Peking Press, 43-56), 1.0 mg/L 2,4-D, 25mM L-proline, 100 mg/L casein hydrolysate, 10 mg/L AgNO₃, 2.5 g/L GELRITE (Schweizerhall, South Plainfield, NJ), and 20 g/L sucrose, with a pH of 5.8. After four to six weeks callus was subcultured onto maintenance medium (initiation medium in which AgNO₃ was omitted and L-proline was reduced to 6 mM). Selection for Type II callus took place for ca. 12-16 weeks.

Plasmid pDAB1834 was transformed into embryogenic callus. For blasting, 140 µg of plasmid DNA was precipitated onto 60 mg of alcohol-rinsed, spherical gold particles (1.5 - 3.0 µm diameter, Aldrich Chemical Co., Inc., Milwaukee, WI) by adding 74 µL of 2.5M CaCl₂ H₂O and 30 µL of 0.1M spermidine (free base) to 300 µL of plasmid DNA and H₂O. The solution was immediately vortexed and the DNA-coated gold particles were allowed to settle. The resulting clear supernatant was removed and the gold particles were resuspended in 1 ml of absolute ethanol. This suspension was diluted with absolute ethanol to obtain 15 mg DNA-coated gold/mL.

Approximately 600 mg of embryogenic callus tissue was spread over the surface of Type II callus maintenance medium as described herein lacking casein hydrolysate and L-proline, but supplemented with 0.2 M sorbitol and 0.2 M mannitol as an osmoticum. Following a 4 h pre-treatment, tissue was transferred to culture dishes containing blasting medium (osmotic media solidified with 20 g/L TC agar (PhytoTechnology Laboratories, LLC, Shawnee Mission, KS) instead of 7 g/L GELRITE. Helium blasting accelerated suspended DNA-coated gold particles towards

and into the prepared tissue targets. The device used was an earlier prototype of that described in US Patent 5,141,131 which is incorporated herein by reference. Tissues were covered with a stainless steel screen (104 μm openings) and placed under a partial vacuum of 25 inches of Hg in the device chamber. The DNA-coated gold particles were further diluted 1:1 with absolute ethanol prior to blasting and were accelerated at the callus targets four times using a helium pressure of 1500 psi, with each blast delivering 20 μL of the DNA/gold suspension. Immediately post-blasting, the tissue was transferred to osmotic media for a 16-24 h recovery period. Afterwards, the tissue was divided into small pieces and transferred to selection medium (maintenance medium lacking casein hydrolysate and L-proline but containing 30 mg/L BASTA® (AgrEvo, Berlin, Germany)). Every four weeks for 3 months, tissue pieces were non-selectively transferred to fresh selection medium. After 7 weeks and up to 22 weeks, callus sectors found proliferating against a background of growth-inhibited tissue were removed and isolated. The resulting BASTA®-resistant tissue was subcultured biweekly onto fresh selection medium. Following western analysis, positive transgenic lines were identified and transferred to regeneration media. Western-negative lines underwent subsequent RNA spot blot analysis to identify negative controls for regeneration.

Regeneration was initiated by transferring callus tissue to cytokinin-based induction medium, which consisted of Murashige and Skoog salts, hereinafter MS salts, and vitamins (Murashige and Skoog, (1962) *Physiol. Plant.* 15: 473-497) 30 g/L sucrose, 100 mg/L myo-inositol, 30 g/L mannitol, 5 mg/L 6-benzylaminopurine, hereinafter BAP, 0.025 mg/L 2,4-D, 30 mg/L BASTA®, and 2.5 g/L GELRITE at pH 5.7. The cultures were placed in low light (125 ft-candles) for one week followed by one

week in high light (325 ft-candles). Following a two week induction period, tissue was non-selectively transferred to hormone-free regeneration medium, which was identical to the induction medium except that it

5 lacked 2,4-D and BAP, and was kept in high light. Small (1.5-3 cm) plantlets were removed and placed in 150x25 mm culture tubes containing SH medium (SH salts and vitamins (Schenk and Hildebrandt, (1972) Can. J. Bot. 50:199-204), 10 g/L sucrose, 100 mg/L myo-inositol, 5 mL/L FeEDTA, and

10 2.5 g/L GELRITE, pH 5.8). Plantlets were transferred to 12 cm pots containing approximately 0.25 kg of METRO-MIX 360 (The Scotts Co. Marysville, OH) in the greenhouse as soon as they exhibited growth and developed a sufficient root system. They were grown with a 16 h photoperiod

15 supplemented by a combination of high pressure sodium and metal halide lamps, and were watered as needed with a combination of three independent Peters Excel fertilizer formulations (Grace-Sierra Horticultural Products Company, Milpitas, CA). At the 6-8 leaf stage, plants

20 were transplanted to five gallon pots containing approximately 4 kg METRO-MIX 360, and grown to maturity.

EXAMPLE 5

Characterization Of Transgenic Maize Plants

25 Expressing Photorhabdus Toxin That Confer Insect Control.

A. Insect Bioassays

A single leaf was sampled from each plant in each test. Eight, 1.4 cm disks were cut from the outer portion of each leaf (approximately 30cm long) avoiding the

30 center vein. Each disk was placed individually into a well of a C-D International 128 well tray (Pitman, NJ.) into which 0.5 ml of a 1.6% aqueous agar solution had been previously pipetted. The solidified agar prevented the leaf disks from drying out. The adaxial surface of

35 the disk was always oriented up.

Five neonate southern corn rootworms, *Diabrotica undecimpunctata howardi*, were placed on each disk and the wells were sealed with vented plastic lids. The assay was held at 27°C and 40% RH. Larval mortality and live-weight data were collected after 3 days. Data were subjected to analysis of variance and Duncan's multiple range test ($\alpha = 0.05$) (Proc GLM, SAS Institute Inc., Cary, NC.). Weight data were transformed using a logarithmic function to correct a correlation between the magnitude of the mean and variance.

TABLE 9

Results of Maize Leaf-disk Test vs SCR

Treatment	Mean % Kill (Duncan's)	Mean Survival Weight (mg) (Duncan's)
1834 - 11	68 A	0.064 A
1834 - 17	44 B	0.098 B
1834 - 15	26 BC	0.127 C
HiII control	13 C	0.161 C

Note: Means followed by the same letter are not significantly different based on Duncan's multiple range test ($\alpha=0.05$). Insect groups weighing less than 0.1 mg were set to 0.03 mg instead of zero to conduct a more conservative analysis. Mortality ($\arcsin(\sqrt{\text{}})$) and weight(\log_{10}) data were transformed for analyses.

The results shown in Table 9 demonstrated that two events expressing TcdA protein were statistically distinct from control lines bioassayed using SCR neonates by mortality and survival weight criteria. These results demonstrated that southern corn rootworm were functionally effected by feeding on maize plants containing and expressing the *tcdA* gene. Those plants from 1834-11 were used to generate progeny for testing of inheritability of transgene.

B. PRODUCTION AND PROGENY TEST OF *tcdA* TRANSGENIC MAIZE

Origin and growth of progeny plants: Sibling plants 1834-11-07 and 1834-11-08, clonally derived by regeneration from the callus of transgenic maize event 1834-11, were transplanted to the greenhouse and pollinated with inbred OQ414. Seeds obtained from these crosses, comprising seed lots 1834-11-07A and 1834-11-08A, were planted in Rootainers (1 ½ inch x 2 inch x 8 inch deep, product #647, C. Hummert Intl., Earth City, Mo.) filled with Metro-Mix 360 soilless mix (Scotts Terra-Lite, available from Hummert Intl.) and top irrigated with Hoagland's nutrient solution. (Hoagland's solution contains 229 ppm nitrogen as nitrate, 24.6 ppm nitrogen as ammonium, 26 ppm P, 157 ppm K, 187 ppm Ca, 49 ppm Mg. and 30 ppm Na.)

Greenhouse conditions for this trial were: 16 hour days, daylight supplemented by metal halide lamps as needed to achieve a minimum of 600 ?Einsteins/cm² PAR, and ambient temperature 30 C days, 22 C nights.

Leaves were sampled for protein determination approximately one week after planting. Leaf bioassays were conducted 2-3 weeks after planting; root bioassays were initiated approximately 3 weeks post planting.

Protein analysis of progeny plants: Protein was extracted from leaf and root samples harvested from transgenic plants, line 1834-11 progenies, and non-transformed plants. Each sample was placed on a 1.6 x 4 cm piece of 3M WhatmanTM paper. The paper was folded lengthwise and inserted in a flexible straw. A volume of 350 µl of an extraction buffer (9.5 ml of 0.2 M NaH₂PO₄, 15.5 ml of 0.2 M Na₂HPO₄, 2 ml of 0.5 M Na₂EDTA, 100 ml of Triton X-100, 1 ml of 10% Sarkosyl, 78 ml of beta-mercaptoethanol, H₂O to bring total volume to 100 ml, 50 µg/ml Antipain, 50 µg/ml Leupeptin, 0.1 mM Chymostatin, 5 µg/ml Pepstatin) was pipetted on to the paper. The straw containing the

sample was then passed through a rolling device used for squeezing the extract into a 1.5 ml microcentrifuge tube. The extract was centrifuged for 10 minutes at 14,000 rpm in an Eppendorf refrigerated micro-centrifuge. The
5 supernatant was transferred into a new tube. The amount of the total extractable protein was determined using a standard BioRad Protein Analysis protocol (BioRad Laboratories, Hercules, CA).

The presence of the TcdA protein was visualized by
10 Western blot analysis following a standard procedure for protein separation (Laemmli, 1970). A volume of twenty μ l of extract was loaded in each well of 4-20% gradient polyacrylamide gel (Owl Scientific Co., MA) for electrophoresis. Subsequently, the protein was
15 transferred onto a nitrocellulose membrane using a semi-dry electroblotter (Pharmacia LKB Biotechnology, Piscataway, NJ). The membrane was incubated for one hour in TBST-M solution (10% milk in TBST solution; 25 mM Tris HCL pH 7.4, 136 mM NaCl, 2.7 mM KCl, 0.1% Tween 20).
20 Thereafter, the primary antibody (Anti-TcdA in TBST-M) was added. After one hour, the membrane was washed with TBST for five minutes, three times. Then the secondary antibody solution (goat anti-rabbit IgG conjugated to horseradish peroxidase; Bio-Rad Laboratories, in TBST-M)
25 was added to the membrane. After one hour of incubation, the membrane was washed with an excess amount of TBST for 10 minutes, four times. The protein was visualized using the Super Signal® West Pico chemiluminescence method (Pierce Chemical Co., Rockford, IL). The protein blot
30 was exposed on a Hyper-film (Amersham, Arlington Heights, IL) and was developed within 3 minutes. The intensity of the protein band was measured using a densitometer (Molecular Dynamics Inc., Sunnyvale, CA) and compared to standards.

35 Three of six plants from seed lot 1834-11-07A and three of six plants from seed lot 1834-11-08A produced

detectable levels of TcdA protein (Table 1).

Approximately 3.8 to 13.3 ppm of TcdA were detected in the leaf blades and 4.1 to 8.4 ppm were detected in the leaf tips of the protein-positive plants. The amounts of
5 TcdA protein detected in the roots were slightly lower than those found in the leaves.

Insect bioassays with progeny plants: Plants were selected for bioassay based on results from Western blot
10 analysis. Twelve (12), 6.4 mm diameter leaf discs were cut from the youngest leaf of each 2 week old seedling. Each disc was placed in a well of a 128-well tray (CD International) containing approximately 0.5mL of a solidified 2% agar in water solution. Two neonate
15 southern corn rootworm, *Diabrotica undecimpunctata howardi* (Barber) (SCR), were placed in each well with a leaf disc. Trays were covered with perforated lids and maintained under a controlled environment for 3 days (28 C; 16 hours light:8 hours dark; approx. 60% relative
20 humidity). Living larvae from 4 leaf discs were pooled and weighed producing 3 weight determinations per plant. Average weights were calculated by dividing the pooled weight by the number of survivors. Differences in
25 average weights of SCR fed leaf discs from protein positive and protein negative plants were assessed using analysis of variance on the natural log-transformed average weights (Minitab, v. 12.2, Minitab Inc., State College, PA).

30 Root bioassays were initiated approximately 1 week after the initiation of the leaf disc bioassays. Approximately 24h prior to eclosion, SCR eggs were suspended in a 0.15% solution of agar in water to a concentration of 100 eggs/ml. Plants were inoculated
35 with SCR eggs by pipetting 2.0 ml of the egg suspension (ie., approximately 200 eggs) just below the soil surface at the base of each plant. Two weeks after inoculation, plants were removed from their Roottrainer pots, their

roots washed free of potting mix, and scored for rootworm damage based on a 1 (resistant) to 9 (susceptible) rating system (Welch, 1977). The results of the root ratings were examined using non-parametric tests to determine if the distribution of root ratings from the protein positive plants was the same as the distribution of the ratings from the protein negative plants. Testing was done at the 5% significance level. (StatXact v.3, CYTEL Software Corporation, Cambridge MA)

10

Results from leaf and root bioassays of *tcdA* protein positive and protein negative progeny plants are summarized in Table 10. The average weights of SCR larvae fed leaf discs from protein positive plants were significantly lower than those of larvae fed leaf discs from protein negative plants ($F = 4.6$; $d.f. = 1, 34$; $P \leq 0.001$. The Kolmogorov-Smirnov 2 sample test ($p=0.04$) and the Wald Wolfowitz runs test ($p=0.001$) indicated that the protein positive and protein negative root rating distributions were not similar. The Wilcoxon- Mann-Whitney test ($p=0.0206$) and the Normal Scores test ($p=0.206$) indicated that the average score for the protein positive plants was lower than the average root rating from the protein negative plants.

25

Table 10. Protein analysis and insect bioassay results with progeny of *TcdA* transgenic maize.

Plant Number	TcdA Protein	Leaf Disc Bioassay Avg. Wt. (mg)	Root Bioassay Root Rating (1-9)
1834-11-07A-30	PRO-	0.190	8
1834-11-08A-21	PRO-	0.196	9
1834-11-08A-16	PRO-	0.195	9
1834-11-08A-14	PRO-	0.137	9
1834-11-07A-22	PRO-	0.208	9
1834-11-07A-20	PRO-	0.175	9

1834-11-07A-26	PRO+	0.118	9
1834-11-08A-17	PRO+	0.132	8
1834-11-07A-14	PRO+	0.110	2
1834-11-07A-11	PRO+	0.106	4
1834-11-08A-28	PRO+	0.129	8
1834-11-08A-27	PRO+	0.108	4

DNA analysis of progeny plants: Leaf samples from 1834-11.7A and 1834-11.8A progeny plants were in conical 50 ml polypropylene tubes and dried in a Labconco Freeze Dry

5 Lyophilizer (Kansas City, MO) for 1-2 days. Lyophilized leaves were then ground in a Tecator Cyclotec 1093 Sample mill grinder (Hoganas, Sweden) and stored at -20C.

Genomic DNA was extracted by the following procedure: (1) to a 25 ml Conical tube containing 300-500 mg of ground

10 tissue, 9 ml of CTAB (cetyl trimethylammonium bromide solution) was added, and incubated at 65°C for 1 hour; (2) 4.5 ml of chloroform: octanol (24:1) was added and mixed gently for 5 minutes; (3) samples were centrifuged at 2000 rpm and DNA was precipitated from the supernatant

15 with an equal volume of isopropanol; (4) DNA was collected on a glass hook, washed in ethanol, and dissolved in TE (10 mM Tris.HCl, 0.5 mM EDTA, pH8.0).

Genomic DNA was digested at 37 °C. for 2 hours in an

20 Eppendorf tube containing the following mixture:

8 µl of 800ug/ml DNA, 2 µl 1 mg/ml BSA (Bovine serum albumin), 2 µl 10x buffer, 1 µl SacI, 1 µl EcoRI, and 6 µl H₂O. Digested DNA samples were electrophoresed overnight at 40 mA in a 0.85% SeaKem LE agarose gel (FMC, Rockland,

25 Maine). The gel was blotted onto Millipore Immobilon-Ny+ (Bedford, MA) membrane overnight in 20X SSC (NaCl 175.2 g/l, Na citrate 88 g/l). The probe DNA was cut with BamHI/SacI (NEB, Beverly, MA) from pDAB1551 plasmid, which released a 7356 bp fragment containing the open

30 reading frame of the rebuilt *tcdA* gene. This 7356 bp fragment was labeled with P32 using a Stratagene Prime-it

RmT dCTP-Labeling Reactions kit (La Jolla, CA) and used for Southern hybridization. Hybridization was conducted in hybridization buffer (10% polyethylene glycol, 7% SDS [Sodium dodecyl sulfate], 0.6X SSC, 10 mM NaPO₄, 5 mM EDTA, 10 µg/ml denatured salmon sperm) at 60 °C overnight. After hybridization, the membrane was washed with 10X SSC plus 0.1% SDS at 60 °C for 30 min and exposed to X ray film (Hyperfilm® MP, Amersham Life Sciences, Piscataway, NJ) for 1-2 days.

10

Results summarized indicate that a pattern of 8 hybridizing bands (the size of the expected fragment and larger) cosegregated with protein expression in 50% of all progeny assayed. These results are characteristic of a complex insertion at a single site. All seedlings containing the insert also expressed toxin protein.

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Example 6

Transformation Of Rice With a Vector Carrying Plasmid pDAB1553 Encoding *Photorhabdus* Toxins

20

A. Plasmid pDAB1553

Plasmid pDAB1553 containing *tcdA* driven by the maize ubiquitin1 promoter and *hpt* (hygromycin phosphotransferase providing resistance to the antibiotic hygromycin) under the control of 35T (a modified 35S promoter), was used for transformation.

25

Preparation of rice transformation vectors was accomplished in two steps. First, a modified plant-optimized *tcdA* coding region was ligated into a rice plant expression cassette plasmid. In this step, the coding region was placed under the transcriptional control of a promoter functional in plant cells. RNA transcription termination and polyadenylation were mediated by a downstream copy of the terminator region from the *Agrobacterium* nopaline synthase gene. One

35

plasmid designed to function in this role is plasmid pDAB1538 (described in the section on maize transformation vectors). In the second step, the complete gene comprised of the promoter, coding region, and terminator region was ligated to a rice plant transformation vector that contained a plant expressible selectable marker gene which allowed the selection of transformed rice plant cells amongst a background of nontransformed cells. An example of such a vector is pDAB354-NotI.

It is a feature of pDAB354-NotI that the hygromycin phosphotransferase protein, which has as its substrate hygromycin B and related compounds, is produced in plant cells through transcription of its coding region mediated by the Cauliflower Mosaic Virus 35S promoter and that termination of transcription plus polyadenylation are mediated by the nopaline synthase terminator region. It is further a feature of pDAB354-NotI that any DNA fragment containing flanking NotI sites can be cloned into the unique NotI site of pDAB354-NotI, thus physically linking the introduced DNA fragment to the aforementioned selectable marker gene.

To prepare a plant-expressible gene to produce the non-targeted TcdA protein in rice plant cells, DNA of a plasmid (pA0H_4-OPTI) containing the plant-optimized *tcdA* coding region, (SEQ ID No:3) was cleaved with restriction enzymes *NcoI* and *SacI*, and the large 7550 bp fragment was ligated to similarly-cut DNA of plasmid pDAB1538 to produce plasmid pDAB1551. DNA of pDAB1551 was then digested with *NotI*, and the large 9933 bp fragment was ligated to *NotI* digested DNA of pDAB354-NotI to produce plasmid pDAB1553.

It is a feature of plasmid pDAB1553 that the *ubil* and 35S promoters are encoded on the same DNA strand.

B. Production of Rice transgenics

For initiation of embryogenic callus, mature seeds of a *Japonica* cultivar, Taipci 309 were dehusked and surface-sterilized in 70% ethanol for 2-5 min. followed by a 30-45 min soak in 50% commercial bleach (2.6% sodium hypochlorite) with a few drops of 'Liquinox' soap. The seeds were then rinsed 3 times in sterile distilled water and placed on filter paper before transferring to 'callus induction' medium (i.e., NB). The NB medium consisted of N6 macro elements (Chu, 1978, The N6 medium and its application to anther culture of cereal crops. Proc. Symp. Plant Tissue Culture, Peking Press, p43-56), B5 micro elements and vitamins (Gamborg et al., 1968, Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151-158), 300 mg/L casein hydrolysate, 500 mg/L L-proline, 500 mg/L L-glutamine, 30 g/L sucrose, 2 mg/L 2,4-dichloro-phenoxyacetic acid (2,4-D), and 2.5 g/L gelrite (Schweizerhall, NJ) with the pH adjusted to 5.8. The mature seed cultured on 'induction' media were incubated in the dark at 28°C. After 3 weeks of culture, the emerging primary callus induced from the scutellar region of mature embryo was transferred to fresh NB medium for further maintenance.

About 140 µg of plasmid pDAB1553 DNA was precipitated onto 60 mg of 1.0 micron (Bio-Rad) gold particles as described herein.

For helium blasting, actively growing embryogenic callus cultures, 2-4 mm in size, were subjected to a high osmoticum treatment. This treatment included placing of callus on NB medium with 0.2 M mannitol and 0.2 M sorbitol (Vain et al., 1993, Osmoticum treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep. 12: 84-88) for 4 h before helium blasting. Following osmoticum treatment, callus cultures were transferred to 'blasting' medium (NB+2% agar) and covered with a stainless steel screen (230 micron). The callus cultures were blasted at

2,000 psi helium pressures twice per target. After blasting, callus was transferred back to the media with high osmoticum overnight before placing on selection medium, which consisted NB medium with 30 mg/L hygromycin. After 2 weeks, the cultures were transferred to fresh selection medium with a higher concentration of selection agent, i.e., NB+50mg/L hygromycin (Li et al., 1993, An improved rice transformation system using the biolistic method. Plant Cell Rep. 12: 250-255).

Compact, white-yellow, embryogenic callus cultures, recovered on NB+50 mg/L hygromycin, were regenerated by transferring to 'pre-regeneration' (PR) medium + 50 mg/L hygromycin. The PR medium consisted of NB medium with 2 mg/L benzyl aminopurine (BAP), 1 mg/L naphthalene acetic acid (NAA), and 5 mg/L abscisic acid (ABA). After 2 weeks of culture in the dark, they were transferred to 'regeneration' (RN) medium. The composition of RN medium is NB medium with 3 mg/L BAP, and 0.5 mg/L NAA. The cultures on RN medium were incubated for 2 weeks at 28° C under high fluorescent light (325-ft-candles). The plantlets with 2 cm shoot were transferred to 1/2 MS medium (Murashige and Skoog, 1962, A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant.15:473-497) with 1/2 B5 vitamins, 10 g/L sucrose, 0.05 mg/L NAA, 50 mg/L hygromycin and 2.5 g/L gelrite adjusted to pH 5.8 in magenta boxes. When plantlets were established with well-developed root systems, they were transferred to soil (1 metromix: 1 top soil) and raised in the greenhouse (29/24°C day/night cycle, 50-60% humidity, 12 h photoperiod) until maturity.

EXAMPLE 7

Characterization Of Transgenic Rice Plants Expressing Photorhabdus Toxin That Confer Insect Control.

A. Insect bioassays

Insect bioassays were performed using leaf discs and shown to be highly effective in controlling Southern corn rootworm. *Diabrotica undecimpunctata howardi* eggs are obtained from French Ag Research and hatched in petri dishes held at 28.5°C and 40% RH. The aerial parts are sampled from the transgenic plants and placed, singly into inverted petri dishes (100x15mm) containing 15ml of 1.6% aqueous agar in the bottom to provide humidity and filter paper in the top to absorb condensation. These preparations are infested with five neonate larvae per dish and held at 28.5°C and 40% RH for 3 days. Mortality and larval weights are recorded. Weight data were transformed using a logarithmic function to correct a correlation between the magnitude of the mean and variance.

Table 11

Treatment	Average Survivor Weight in mg ¹ (Duncan's Grouping)	Presence TcdA greenhouse-grown plants (number of +/-number of plants tested)
GUS Control	0.390 A	-
1553-33	0.170 BCD	++
1553-44	0.167 BCD	+++
1553-62	0.125 CD	+++
1553-41	0.100 D	+++

Note: Means followed by the same letter are not significantly different based on Duncan's multiple range test (alpha=0.05).

Insect groups weighing less than 0.1 mg were set to 0.03 mg instead of zero to conduct a more conservative analysis. Weight data were transformed (Log10) for analyses. A single replicate was used on each of three test dates. Plants were sampled from magenta boxes. The results demonstrate that in leaf disc bioassays, several rice events derived by transformation with *tcdA* gene were demonstrated to statistically have a functional affect on corn rootworm neonate.

Claims

1. An isolated nucleic acid of SEQ ID NO: 3 or SEQ ID NO:4.
2. A transgenic monocot cell having a genome comprising
5 SEQ ID NO:3 or SEQ ID NO:4.
3. A transgenic dicot cell having a genome comprising SEQ ID NO:3 or SEQ ID NO:4.
4. A transgenic plant with a genome comprising a
nucleic acid of SEQ ID NO: 3 or SEQ ID NO:4 that imparts
10 insect resistance.
5. A transgenic plant of claim 4 wherein the plant is rice.
6. A transgenic plant of claim 4 wherein the plant is maize.
- 15 7. A transgenic plant of claim 4 wherein the plant is tobacco.

SEQUENCE LISTING

<110> Petell, Jim
 Merlo, Donald
 Herman, Rod
 Roberts, Jean
 Guo, Lining
 Schafer, Barry
 Sukhapinda, Kitisri
 Owens Merlo, Ann

<120> Transgenic Plants Expressing Photorhabdus Toxin

<130> 50698

<140>

<141>

<150> US 60/148,356

<151> 1999-08-11

<160> 8

<170> PatentIn Ver. 2.0

<210> 1

<211> 7551

<212> DNA

<213> Photorhabdus luminescens

<220>

<221> CDS

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Gly Phe Asn Cys Leu Thr Asp Ile Ser His Ser Ser Phe Asn Glu Phe	
20 25 30	
cgc cag caa gta tct gag cac ctc tcc tgg tcc gaa aca cac gac tta	144
Arg Gln Gln Val Ser Glu His Leu Ser Trp Ser Glu Thr His Asp Leu	
35 40 45	
tat cat gat gca caa cag gca caa aag gat aat cgc ctg tat gaa gcg	192
Tyr His Asp Ala Gln Gln Ala Gln Lys Asp Asn Arg Leu Tyr Glu Ala	
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Arg Ile Leu Lys Arg Ala Asn Pro Gln Leu Gln Asn Ala Val His Leu	
65 70 75 80	
gcc att ctc gct ccc aat gct gaa ctg ata ggc tat aac aat caa ttt	288
Ala Ile Leu Ala Pro Asn Ala Glu Leu Ile Gly Tyr Asn Asn Gln Phe	
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agc ggt aga gcc agt caa tat gtt gcg ccg ggt acc gtt tct tcc atg	336
Ser Gly Arg Ala Ser Gln Tyr Val Ala Pro Gly Thr Val Ser Ser Met	

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aat gct tat caa atg gat gtg gag cta ttt ccc ttc ggt ggt gag aat Asn Ala Tyr Gln Met Asp Val Glu Leu Phe Pro Phe Gly Gly Glu Asn 340 345 350			1056

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Ser Ile Lys Leu Asn Asp Lys Arg Glu Leu Val Arg Thr Glu Gly Ala	
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Gly Ser Trp Ala Tyr Ala Ala Ala Lys Phe Thr Val Glu Glu Tyr Asn	
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Gln Tyr Ser Phe Leu Leu Lys Leu Asn Lys Ala Ile Arg Leu Ser Arg	
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Ala Thr Glu Leu Ser Pro Thr Ile Leu Glu Gly Ile Val Arg Ser Val	
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Asn Leu Gln Leu Asp Ile Asn Thr Asp Val Leu Gly Lys Val Phe Leu	
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Thr Lys Tyr Tyr Met Gln Arg Tyr Ala Ile His Ala Glu Thr Ala Leu	
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Ile Leu Cys Asn Ala Pro Ile Ser Gln Arg Ser Tyr Asp Asn Gln Pro	
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Phe Ser Thr Gly Asp Glu Glu Ile Asp Leu Asn Ser Gly Ser Thr Gly	
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Lys Ile Lys Asn Asn Leu Lys Asn L u Ser Asn Leu Tyr Ile Gly Lys	
580 585 590	

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595 600 605	
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Leu Ile Ala Val Gly Glu Gly Lys Thr Asn Leu Ser Ala Ile Ser Asp	
610 615 620	
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625 630 635 640	
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Lys Tyr Thr Pro Gly Ser Ser Glu Ala Val Glu Thr Gln Glu His Ile	
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Val Gln Tyr Cys Gln Ala Leu Ala Gln Leu Glu Met Val Tyr His Ser	
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Thr Gly Ile Asn Glu Asn Ala Phe Arg Leu Phe Val Thr Lys Pro Glu	
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850 855 860	
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Val Asn Val Ala Gln Gln Leu Asn Val Ala Pro Gln Gly Val Ser Ala	
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900 905 910	
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930 935 940	
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945 950 955 960	
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Val Ser Ala Ala Ile Lys Thr Thr Arg Ile Ala Glu Ala Ile Ala Ser	
980 985 990	
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 1505 1510 1515 1520

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 1780 1785 1790

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 Asn Arg Trp Leu Lys Tyr Val Trp Ser Pro Ser Gly Tyr Ile Val His
 1795 1800 1805

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cca cga cta gac aga gcc gcg gat atc act acc caa aat gct cac gac Pro Arg Leu Asp Arg Ala Ala Asp Ile Thr Thr Gln Asn Ala His Asp 1905 1910 1915 1920	5760
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Gln Leu Thr Cys Pro Ala Glu Ile Ala Leu Tyr Pro Phe Asp Thr Phe	
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Arg Glu Lys Thr Arg Gly Met Val Asn Trp Gly Glu Ala Lys Arg Ile	
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Tyr Glu Ile Ala Gln Ala Glu Gln Asp Arg Asn Leu Leu His Glu Lys	
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Gly Thr Arg Gln Met Leu Gly Phe Ile Gln Gly Tyr Ser Asp Leu Phe	
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Gly Asn Arg Ala Asp Asn Tyr Ala Ala Pro Gly Ser Val Ala Ser Met	
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Phe Ser Pro Ala Ala Tyr Leu Thr Glu Leu Tyr Arg Glu Ala Lys Asn	
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Leu His Asp Ser Ser Ser Ile Tyr Tyr Leu Asp Lys Arg Arg Pro Asp	
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Thr Leu Ala Leu Ser Asn Glu Leu Cys Leu Ala Gly Ile Glu Thr Lys	
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Leu Ser Gly Glu Thr Pro Tyr His His Ala Tyr Glu Thr Val Arg Glu	
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Ile His Asn Leu Thr Ile Ala Glu Leu Asn Ile Leu Leu Val Ile Cys	
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Lys Ile Val Glu Thr Leu Leu Trp Ile Thr Gln Trp Leu Lys Thr Gln	
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Thr Thr Leu Thr Pro Glu Ile Ser Asn Leu Thr Ala Thr Leu Ser Ser	
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	805	810	815
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Gln His Ala Ser Leu Ile Leu Ala Ala Leu Lys Asp Gly Ala Leu Thr			
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Gln Thr Val Val Lys Val Phe Leu Ser Tyr Phe Ile Glu Ala Thr Gly				
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Asn Lys Asn His Leu Trp Val Arg Ala Lys Tyr Gln Lys Glu Thr Thr				
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Phe Leu Ser Asp Asp His Lys Thr Phe Ser Gly Leu Ser Ser Ala Gln				
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Leu Tyr Phe Trp Glu Leu Phe Tyr Tyr Thr Pro Met Met Met Ala His				
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Arg Leu Leu Gln Glu Gln Asn Phe Asp Ala Ala Asn His Trp Phe Arg				
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Tyr Val Trp Ser Pro Ser Gly Tyr Ile Val Asp Gly Lys Ile Ala Ile				
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Tyr His Trp Asn Val Arg Pro Leu Glu Glu Asp Thr Ser Trp Asn Ala				
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Gln Gln Leu Asp Ser Thr Asp Pro Asp Ala Val Ala Gln Asp Asp Pro				
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Met His Tyr Lys Val Ala Thr Phe Met Ala Thr Leu Asp Leu Leu Met				
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Ala Arg Gly Asp Ala Ala Tyr Arg Gln Leu Glu Arg Asp Thr Leu Ala				
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Pro Gln Val Met Leu Ser Thr Thr Trp Ala Asn Pro Thr Leu Gly Asn				
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Pro Lys Gly Cys Ser Ala Leu Ala Val Ser His Gly Thr Asn Asp Ser
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Gly Gln Phe Gln Leu Asp Phe Asn Asp Gly Lys Tyr Leu Pro Phe Glu
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Gly Ile Ala Leu Asp Asp Gln Gly Thr Leu Asn Leu Gln Phe Pro Asn
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Gln Cys Gly Phe Asn Cys Leu Thr Asp Ile Ser His Ser Ser Phe Asn
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gag ttc aga caa caa gtc tct gag cac ctc tcc tgg tcc gag acc cat   143
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gac ctc tac cat gac gct cag caa gct cag aag gac aac agg ctc tac   191
Asp Leu Tyr His Asp Ala Gln Gln Ala Gln Lys Asp Asn Arg Leu Tyr
      50                      55                      60

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Gln Phe Ser Gly Arg Ala Ser Gln Tyr Val Ala Pro Gly Thr Val Ser	
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Ser Met Phe Ser Pro Ala Ala Tyr Leu Thr Glu Leu Tyr Arg Glu Ala	
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Arg Asn Leu His Ala Ser Asp Ser Val Tyr Tyr Leu Asp Thr Arg Arg	
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Leu Ser Thr Leu Ser Leu Ser Asn Glu Leu Leu Leu Glu Ser Ile Lys	
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Thr Glu Ser Lys Leu Glu Asn Tyr Thr Lys Val Met Glu Met Leu Ser	
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Val Arg Glu Val Ile Gln Leu Gln Asp Pro Gly Leu Glu Gln Leu Asn	
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Ile Asn Ala Ser Ile Ser Pro Glu Leu Phe Asn Ile Leu Thr Glu Glu	
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Pro Ser Gly Ser Trp Ala Tyr Ala Ala Lys Phe Thr Val Glu Glu	
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Asp Val Ser Leu Phe Arg Leu Leu Lys Ile Thr Asp His Asp Asn Lys	
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Ser Asp Lys Gln Leu Ala Thr Leu Ile Arg Lys Leu Asn Thr Ile Thr	
625 630 635	
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Ser Trp Leu His Thr Gln Lys Trp Ser Val Phe Gln Leu Phe Ile Met	
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Thr Ala Glu Gln Leu Ala Asp Ala Met Asn Leu Asp Ala Asn Leu Leu	835	840	845	
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Ala Ala Ile Lys Ser Arg Asp Asp Leu Tyr Gln Tyr Leu Leu Ile Asp	960	965	970	975
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Tyr Tyr Pro Glu Asn Tyr Ile Asp Pro Thr Met Arg Ile Gly Gln Thr	1040	1045	1050	1055

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Asn Asp Gln Gly Leu Thr Tyr Phe Ile Gly Leu Ser Glu Thr Asp Ala	
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Gly Glu Tyr Tyr Trp Arg Ser Val Asp His Ser Lys Phe Asn Asp Gly	
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Lys Phe Ala Ala Asn Ala Trp Ser Glu Trp His Lys Ile Asp Cys Pro	
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Ile Asn Pro Tyr Lys Ser Thr Ile Arg Pro Val Ile Tyr Lys Ser Arg	
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Leu Tyr Leu Leu Trp Leu Glu Gln Lys Glu Ile Thr Lys Gln Thr Gly	
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Asn Ser Lys Asp Gly Tyr Gln Thr Glu Thr Asp Tyr Arg Tyr Glu Leu	
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Lys Leu Ala His Ile Arg Tyr Asp Gly Thr Trp Asn Thr Pro Ile Thr	
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Phe Asp Val Asn Lys Lys Ile Ser Glu Leu Lys Leu Glu Lys Asn Arg	
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Ala Pro Gly Leu Tyr Cys Ala Gly Tyr Gln Gly Glu Asp Thr Leu Leu	
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Val Met Phe Tyr Asn Gln Gln Asp Thr Leu Asp Ser Tyr Lys Asn Ala	
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Ser Met Gln Gly Leu Tyr Ile Phe Ala Asp Met Ala Ser Lys Asp Met	
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Thr Pro Glu Gln Ser Asn Val Tyr Arg Asp Asn Ser Tyr Gln Gln Phe	
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 Asp Thr Asn Asn Val Arg Arg Val Asn Asn Arg Tyr Ala Glu Asp Tyr
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gag atc cca agc tct gtc agc tct cgc aag gac tac ggc tgg ggt gac 3983
 Glu Ile Pro Ser Ser Val Ser Ser Arg Lys Asp Tyr Gly Trp Gly Asp
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tac tac ctc agc atg gtg tac aac ggt gac atc cca acc atc aac tac 4031
 Tyr Tyr Leu Ser Met Val Tyr Asn Gly Asp Ile Pro Thr Ile Asn Tyr
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 Lys Ala Ala Ser Ser Asp Leu Lys Ile Tyr Ile Ser Pro Lys Leu Arg
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 Ile Ile His Asn Gly Tyr Glu Gly Gln Lys Arg Asn Gln Cys Asn Leu
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 Met Asn Lys Tyr Gly Lys Leu Gly Asp Lys Phe Ile Val Tyr Thr Ser
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ctt ggt gtc aac cca aac aac agc tcc aac aag ctc atg ttc tac cca 4223
 Leu Gly Val Asn Pro Asn Asn Ser Ser Asn Lys Leu Met Phe Tyr Pro
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 Val Tyr Gln Tyr Ser Gly Asn Thr Ser Gly Leu Asn Gln Gly Arg Leu
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 Leu Phe His Arg Asp Thr Thr Tyr Pro Ser Lys Val Glu Ala Trp Ile
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 Pro Gly Ala Lys Arg Ser Leu Thr Asn Gln Asn Ala Ala Ile Gly Asp
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 Asp Tyr Ala Thr Asp Ser Leu Asn Lys Pro Asp Asp Leu Lys Gln Tyr
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 Ile Phe Met Thr Asp Ser Lys Gly Thr Ala Thr Asp Val Ser Gly Pro
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 Val Glu Ile Asn Thr Ala Ile Ser Pro Ala Lys Val Gln Ile Ile Val
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 Lys Ala Gly Gly Lys Glu Gln Thr Phe Thr Ala Asp Lys Asp Val Ser
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 Ile Gln Pro Ser Pro Ser Phe Asp Glu Met Asn Tyr Gln Phe Asn Ala
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Leu Glu Ile Asp Gly Ser Gly L u Asn Phe Ile Asn Asn Ser Ala Ser
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 Ile Asp Val Thr Phe Thr Ala Phe Ala Glu Asp Gly Arg Lys Leu Gly
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 Tyr Glu Ser Phe Ser Ile Pro Val Thr Leu Lys Val Ser Thr Asp Asn
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gca ctc acc ctt cat cac aac gag aac ggt gct cag tac atg caa tgg 4799
 Ala Leu Thr Leu His His Asn Glu Asn Gly Ala Gln Tyr Met Gln Trp
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 Gln Ser Tyr Arg Thr Arg Leu Asn Thr Leu Phe Ala Arg Gln Leu Val
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 Ala Arg Ala Thr Thr Gly Ile Asp Thr Ile Leu Ser Met Glu Thr Gln
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 Ile Pro Pro Tyr Asn Leu Ser Thr His Gly Asp Glu Arg Trp Phe Lys
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 1665 1670 1675

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 Gly Gln Leu Thr Asp Thr Asn Ile Asn Ile Thr Leu Phe Ile Pro Leu
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 Asp Asp Val Pro Leu Asn Gln Asp Tyr His Ala Lys Val Tyr Met Thr
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 Phe Lys Lys Ser Pro Ser Asp Gly Thr Trp Trp Gly Pro His Phe Val
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cgt gat gac aag ggc atc gtc acc atc aac cca aag tcc atc ctc acc 5231
 Arg Asp Asp Lys Gly Ile Val Thr Ile Asn Pro Lys Ser Ile Leu Thr
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 His Phe Glu Ser Val Asn Val Leu Asn Asn Ile Ser Ser Glu Pro Met
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 Asp Phe Ser Gly Ala Asn Ser Leu Tyr Phe Trp Glu Leu Phe Tyr Tyr
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 Thr Pro Met Leu Val Ala Gln Arg Leu Leu His Glu Gln Asn Phe Asp

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Glu Ala Asn Arg Trp Leu Lys Tyr Val Trp Ser Pro Ser Gly Tyr Ile			
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Val His Gly Gln Ile Gln Asn Tyr Gln Trp Asn Val Arg Pro Leu Leu			
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Glu Asp Thr Ser Trp Asn Ser Asp Pro Leu Asp Ser Val Asp Pro Asp			
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Ala Val Ala Gln His Asp Pro Met His Tyr Lys Val Ser Thr Phe Met			
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agg acc ttg gac ctc ttg att gcc aga ggt gac cat gct tac cgc caa			5615
Arg Thr Leu Asp Leu Leu Ile Ala Arg Gly Asp His Ala Tyr Arg Gln			
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Leu Glu Arg Asp Thr Leu Asn Glu Ala Lys Met Trp Tyr Met Gln Ala			
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Ser Asp Pro Arg Leu Asp Arg Ala Ala Asp Ile Thr Thr Gln Asn Ala			
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cca ctc tcc ctc aga tct gct aac acc ctc act gac ttg ttc ctc cca			5855
Pro Leu Ser Leu Arg Ser Ala Asn Thr Leu Thr Asp Leu Phe Leu Pro			
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Gln Ile Asn Glu Val Met Met Asn Tyr Trp Gln Thr Leu Ala Gln Arg			
1955	1960	1965	
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Val Tyr Asn Leu Arg His Asn Leu Ser Ile Asp Gly Gln Pro Leu Tyr			
1970	1975	1980	
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Leu Pro Ile Tyr Ala Thr Pro Ala Asp Pro Lys Ala Leu Leu Ser Ala			
1985	1990	1995	
gct gtg gct acc agc caa ggt ggt ggc aag ctc cca gag tcc ttc atg			6047
Ala Val Ala Thr Ser Gln Gly Gly Gly Lys Leu Pro Glu Ser Phe Met			
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Ser Leu Trp Arg Phe Pro His Met Leu Glu Asn Ala Arg Gly Met Val			
2020	2025	2030	

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caa gat gct gag gct ctc aac gct ttg ctc cag aac cag gca gct gag Gln Asp Ala Glu Ala Leu Asn Ala Leu Leu Gln Asn Gln Ala Ala Glu 2050 2055 2060	6191
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gat gct gag aag aca gtc ctt gag aag agc aag gct ggt gcc caa tct Asp Ala Glu Lys Thr Val Leu Glu Lys Ser Lys Ala Gly Ala Gln Ser 2080 2085 2090 2095	6287
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Lys Leu Gln Leu Thr Cys Pro Ala Glu Ile Ala Leu Tyr Pro Phe Asp	
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Thr Phe Arg Glu Lys Thr Arg Gly Met Val Asn Trp Gly Glu Ala Lys	
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Arg Ile Tyr Glu Ile Ala Gln Ala Glu Gln Asp Arg Asn Leu Leu His	
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Glu Lys Arg Ile Phe Ala Tyr Ala Asn Pro Leu Leu Lys Asn Ala Val	
65 70 75	
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Arg Leu Gly Thr Arg Gln Met Leu Gly Phe Ile Gln Gly Tyr Ser Asp	
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Leu Phe Gly Asn Arg Ala Asp Asn Tyr Ala Ala Pro Gly Ser Val Ala	
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Ser Met Phe Ser Pro Ala Ala Tyr Leu Thr Glu Leu Tyr Arg Glu Ala	
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Lys Asn Leu His Asp Ser Ser Ser Ile Tyr Tyr Leu Asp Lys Arg Arg	
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Pro Asp Leu Ala Ser Leu Met Leu Ser Gln Lys Asn Met Asp Glu Glu	
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Ile Ser Thr Leu Ala Leu Ser Asn Glu Leu Cys Leu Ala Gly Ile Glu	
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Tyr Arg Leu Ser Gly Glu Thr Pro Tyr His His Ala Tyr Glu Thr Val	
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Arg Glu Ile Val His Glu Arg Asp Pro Gly Phe Arg His Leu Ser Gln	
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Ala Pro Ile Val Ala Ala Lys Leu Asp Pro Val Thr Leu Leu Gly Ile	
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Ser Ser His Ile Ser Pro Glu Leu Tyr Asn Leu Leu Ile Glu Glu Ile	
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Pro Glu Lys Asp Glu Ala Ala Leu Asp Thr Leu Tyr Lys Thr Asn Phe	
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Gly Asp Ile Thr Thr Ala Gln Leu Met Ser Pro Ser Tyr Leu Ala Arg	
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Tyr Tyr Gly Val Ser Pro Glu Asp Ile Ala Tyr Val Thr Thr Ser Leu	
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Ser His Val Gly Tyr Ser Ser Asp Ile Leu Val Ile Pro Leu Val Asp	
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Gln Ala Thr Ile Lys Arg Ser Asp Ser Asp Asn Ile L u Ser Ile Gly	
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Lys	Ile	Asp	Gln	Tyr	Ser	Pro	Lys	Ala	Phe	Leu	Leu	Lys	Met	Asn	Lys		
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Ala	Ile	Arg	Leu	Leu	Lys	Ala	Thr	Gly	Leu	Ser	Phe	Ala	Thr	Leu	Glu		
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Arg	Ile	Val	Asp	Ser	Val	Asn	Ser	Thr	Lys	Ser	Ile	Thr	Val	Glu	Val		
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Ala	Val	Gly	Asn	Gln	Leu	Ser	Gln	Phe	Glu	Gln	Leu	Phe	Asn	His	Pro		
			515					520					525				
cca	ctc	aac	ggc	atc	cgc	tac	gag	atc	agc	gag	gac	aac	tcc	aag	cac	1631	
Pro	Leu	Asn	Gly	Ile	Arg	Tyr	Glu	Ile	Ser	Glu	Asp	Asn	Ser	Lys	His		
		530					535					540					
ctc	cca	aac	cca	gac	ctc	aac	ctc	aag	cca	gac	tcc	act	ggt	gat	gac	1679	
Leu	Pro	Asn	Pro	Asp	Leu	Asn	Leu	Lys	Pro	Asp	Ser	Thr	Gly	Asp	Asp		
	545					550					555						
caa	agg	aag	gct	gtc	ctc	aag	agg	gct	ttc	caa	gtc	aac	gct	tct	gag	1727	
Gln	Arg	Lys	Ala	Val	Leu	Lys	Arg	Ala	Phe	Gln	Val	Asn	Ala	Ser	Glu		
560					565					570				575			
ctt	tac	caa	atg	ctc	ttg	atc	act	gac	agg	aag	gag	gat	ggt	gtc	atc	1775	
Leu	Tyr	Gln	Met	Leu	Leu	Ile	Thr	Asp	Arg	Lys	Glu	Asp	Gly	Val	Ile		
				580				585					590				
aag	aac	aac	ttg	gag	aac	ctc	tct	gac	ctc	tac	ctt	gtc	tcc	ctc	ttg	1823	
Lys	Asn	Asn	Leu	Glu	Asn	Leu	Ser	Asp	Leu	Tyr	Leu	Val	Ser	Leu	Leu		
			595					600					605				
gcc	caa	atc	cac	aac	ttg	acc	att	gct	gag	ttg	aac	atc	ctc	ttg	gtc	1871	
Ala	Gln	Ile	His	Asn	Leu	Thr	Ile	Ala	Glu	Leu	Asn	Ile	Leu	Leu	Val		
		610					615					620					
atc	tgc	ggt	tac	ggt	gac	acc	aac	atc	tac	caa	atc	act	gac	gac	aac	1919	
Ile	Cys	Gly	Tyr	Gly	Asp	Thr	Asn	Ile	Tyr	Gln	Ile	Thr	Asp	Asp	Asn		
	625				630						635						
ctt	gcc	aag	att	gtg	gag	acc	ctc	ttg	tgg	atc	acc	caa	tgg	ctc	aag	1967	
Leu	Ala	Lys	Ile	Val	Glu	Thr	Leu	Leu	Trp	Ile	Thr	Gln	Trp	Leu	Lys		
640					645				650					655			
acc	cag	aag	tgg	act	gtc	aca	gac	ctc	ttc	ctc	atg	acc	act	gcc	acc	2015	
Thr	Gln	Lys	Trp	Thr	Val	Thr	Asp	Leu	Ph	Leu	Met	Thr	Thr	Ala	Thr		

660	665	670	
tac tcc acc act ctc act cca gag att tcc aac ctc act gcc acc ctc			2063
Tyr Ser Thr Thr Leu Thr Pro Glu Ile Ser Asn Leu Thr Ala Thr Leu			
675	680	685	
agc tcc acc ctc cac ggc aag gag tcc ctc att ggt gag gac ctc aag			2111
Ser Ser Thr Leu His Gly Lys Glu Ser Leu Ile Gly Glu Asp Leu Lys			
690	695	700	
agg gca atg gct cca tgc ttc acc tct gct ctc cac ctc acc tcc caa			2159
Arg Ala Met Ala Pro Cys Phe Thr Ser Ala Leu His Leu Thr Ser Gln			
705	710	715	
gag gtg gct tac gac ctc ctt ctc tgg att gac caa atc caa cca gct			2207
Glu Val Ala Tyr Asp Leu Leu Leu Trp Ile Asp Gln Ile Gln Pro Ala			
720	725	730	735
caa atc act gtg gat ggt ttc tgg gag gag gtc caa acc act cca acc			2255
Gln Ile Thr Val Asp Gly Phe Trp Glu Glu Val Gln Thr Thr Pro Thr			
740	745	750	
tcc ctc aag gtc atc acc ttc gct caa gtc ttg gct caa ctc tcc ctc			2303
Ser Leu Lys Val Ile Thr Phe Ala Gln Val Leu Ala Gln Leu Ser Leu			
755	760	765	
atc tac aga agg att ggt ctc tct gag act gag ttg tcc ctc att gtc			2351
Ile Tyr Arg Arg Ile Gly Leu Ser Glu Thr Glu Leu Ser Leu Ile Val			
770	775	780	
acc caa tcc agc ctc ttg gtc gct ggc aag tcc atc ctt gat cat ggt			2399
Thr Gln Ser Ser Leu Leu Val Ala Gly Lys Ser Ile Leu Asp His Gly			
785	790	795	
ctc ttg acc ctc atg gct ctt gag ggt ttc cac acc tgg gtc aac ggt			2447
Leu Leu Thr Leu Met Ala Leu Glu Gly Phe His Thr Trp Val Asn Gly			
800	805	810	815
ttg ggt caa cat gct tcc ctc atc ttg gct gca ctc aag gat ggt gct			2495
Leu Gly Gln His Ala Ser Leu Ile Leu Ala Ala Leu Lys Asp Gly Ala			
820	825	830	
ctc acc gtc acc gat gtg gct caa gcc atg aac aag gag gag tcc ctc			2543
Leu Thr Val Thr Asp Val Ala Gln Ala Met Asn Lys Glu Glu Ser Leu			
835	840	845	
ttg caa atg gct gcc aac cag gtg gag aag gac ctc acc aag ctc acc			2591
Leu Gln Met Ala Ala Asn Gln Val Glu Lys Asp Leu Thr Lys Leu Thr			
850	855	860	
tcc tgg acc caa atc gat gcc atc ctc caa tgg ctc caa atg tcc tct			2639
Ser Trp Thr Gln Ile Asp Ala Ile Leu Gln Trp Leu Gln Met Ser Ser			
865	870	875	
gct ctt gct gtc agc cca ttg gac ctt gct ggc atg atg gct ctc aag			2687
Ala Leu Ala Val Ser Pro Leu Asp Leu Ala Gly Met Met Ala Leu Lys			
880	885	890	895
tac ggc att gat cac aac tac gct gcc tgg caa gca gct gcc gct gcc			2735
Tyr Gly Ile Asp His Asn Tyr Ala Ala Trp Gln Ala Ala Ala Ala Ala			
900	905	910	

ctc atg gct gac cat gcc aac cag gct cag aag aag ttg gat gag acc	2783
Leu Met Ala Asp His Ala Asn Gln Ala Gln Lys Lys Leu Asp Glu Thr	
915 920 925	
ttc tcc aag gct ctc tgc aac tac tac atc aac gcc gtg gtt gac tct	2831
Phe Ser Lys Ala Leu Cys Asn Tyr Tyr Ile Asn Ala Val Val Asp Ser	
930 935 940	
gct gcc ggt gtc agg gac agg aac ggt ctc tac acc tac ctc ttg att	2879
Ala Ala Gly Val Arg Asp Arg Asn Gly Leu Tyr Thr Tyr Leu Leu Ile	
945 950 955	
gac aac cag gtc tct gct gat gtc atc acc tcc aga att gct gag gcc	2927
Asp Asn Gln Val Ser Ala Asp Val Ile Thr Ser Arg Ile Ala Glu Ala	
960 965 970 975	
att gct ggc atc caa ctc tac gtc aac agg gct ctc aac agg gat gag	2975
Ile Ala Gly Ile Gln Leu Tyr Val Asn Arg Ala Leu Asn Arg Asp Glu	
980 985 990	
ggt cag ttg gct tct gat gtc tcc acc agg caa ttc ttc acc gac tgg	3023
Gly Gln Leu Ala Ser Asp Val Ser Thr Arg Gln Phe Phe Thr Asp Trp	
995 1000 1005	
gag agg tac aac aag agg tac tcc acc tgg gct ggt gtc tct gag ttg	3071
Glu Arg Tyr Asn Lys Arg Tyr Ser Thr Trp Ala Gly Val Ser Glu Leu	
1010 1015 1020	
gtc tac tac cca gag aac tac gtg gac cca acc caa agg att ggt cag	3119
Val Tyr Tyr Pro Glu Asn Tyr Val Asp Pro Thr Gln Arg Ile Gly Gln	
1025 1030 1035	
acc aag atg atg gat gct ttg ctc caa tcc atc aac cag tcc caa ctc	3167
Thr Lys Met Met Asp Ala Leu Leu Gln Ser Ile Asn Gln Ser Gln Leu	
1040 1045 1050 1055	
aac gct gac act gtg gag gat gct ttc aag acc tac ctc acc tcc ttc	3215
Asn Ala Asp Thr Val Glu Asp Ala Phe Lys Thr Tyr Leu Thr Ser Phe	
1060 1065 1070	
gag caa gtg gcc aac ctc aag gtc atc tct gct tac cat gac aac gtc	3263
Glu Gln Val Ala Asn Leu Lys Val Ile Ser Ala Tyr His Asp Asn Val	
1075 1080 1085	
aac gtg gac caa ggt ctc acc tac ttc att ggc att gac caa gcc gct	3311
Asn Val Asp Gln Gly Leu Thr Tyr Phe Ile Gly Ile Asp Gln Ala Ala	
1090 1095 1100	
cct ggc acc tac tac tgg agg tct gtg gac cac tcc aag tgc gag aac	3359
Pro Gly Thr Tyr Tyr Trp Arg Ser Val Asp His Ser Lys Cys Glu Asn	
1105 1110 1115	
ggc aag ttc gct gcc aac gct tgg ggt gag tgg aac aag atc acc tgc	3407
Gly Lys Phe Ala Ala Asn Ala Trp Gly Glu Trp Asn Lys Ile Thr Cys	
1120 1125 1130 1135	
gct gtc aac cct tgg aag aac atc atc agg cca gtg gtc tac atg tcc	3455
Ala Val Asn Pro Trp Lys Ser Ile Ile Arg Pro Val Val Tyr Met Ser	
1140 1145 1150	

aga ctc tac ttg ctc tgg ctt gag caa cag tcc aag aag tct gat gac 3503
 Arg Leu Tyr Leu L u Trp L u Glu Gln Gln Ser Lys Lys S r Asp Asp
 1155 1160 1165

ggc aag aca act atc tac cag tac aac ctc aag ttg gct cac atc cgc 3551
 Gly Lys Thr Thr Ile Tyr Gln Tyr Asn Leu Lys Leu Ala His Ile Arg
 1170 1175 1180

tac gat ggt tcc tgg aac act cca ttc acc ttc gat gtc act gag aag 3599
 Tyr Asp Gly Ser Trp Asn Thr Pro Phe Thr Phe Asp Val Thr Glu Lys
 1185 1190 1195

gtc aag aac tac acc tcc agc act gat gca gct gag tcc ctt ggt ctc 3647
 Val Lys Asn Tyr Thr Ser Ser Thr Asp Ala Ala Glu Ser Leu Gly Leu
 1200 1205 1210 1215

tac tgc act ggt tac caa ggt gag gac acc ctc ttg gtc atg ttc tac 3695
 Tyr Cys Thr Gly Tyr Gln Gly Glu Asp Thr Leu Leu Val Met Phe Tyr
 1220 1225 1230

tcc atg caa tcc agc tac tcc agc tac act gac aac aac gct cca gtc 3743
 Ser Met Gln Ser Ser Tyr Ser Ser Tyr Thr Asp Asn Asn Ala Pro Val
 1235 1240 1245

act ggt ctc tac atc ttc gct gac atg tcc tct gac aac atg acc aac 3791
 Thr Gly Leu Tyr Ile Phe Ala Asp Met Ser Ser Asp Asn Met Thr Asn
 1250 1255 1260

gct caa gcc acc aac tac tgg aac aac tcc tac cca caa ttc gac act 3839
 Ala Gln Ala Thr Asn Tyr Trp Asn Asn Ser Tyr Pro Gln Phe Asp Thr
 1265 1270 1275

gtc atg gct gac cca gac tct gac aac aag aag gtc atc acc agg cgt 3887
 Val Met Ala Asp Pro Asp Ser Asp Asn Lys Lys Val Ile Thr Arg Arg
 1280 1285 1290 1295

gtc aac aac cgc tac gct gag gac tac gag atc cca agc tct gtc acc 3935
 Val Asn Asn Arg Tyr Ala Glu Asp Tyr Glu Ile Pro Ser Ser Val Thr
 1300 1305 1310

tcc aac agc aac tac tcc tgg ggt gac cac tcc ctc acc atg ctc tac 3983
 Ser Asn Ser Asn Tyr Ser Trp Gly Asp His Ser Leu Thr Met Leu Tyr
 1315 1320 1325

ggt ggc tct gtc cca aac atc acc ttc gag tct gca gct gag gac ctc 4031
 Gly Gly Ser Val Pro Asn Ile Thr Phe Glu Ser Ala Ala Glu Asp Leu
 1330 1335 1340

agg ctc tcc acc aac atg gct ctc tcc atc att cac aac ggt tac gct 4079
 Arg Leu Ser Thr Asn Met Ala Leu Ser Ile Ile His Asn Gly Tyr Ala
 1345 1350 1355

ggc acc agg cgc atc caa tgc aac ctc atg aag caa tac gct tcc ctt 4127
 Gly Thr Arg Arg Ile Gln Cys Asn Leu Met Lys Gln Tyr Ala Ser Leu
 1360 1365 1370 1375

ggt gac aag ttc att atc tac gac tcc agc ttc gat gac gcc aac agg 4175
 Gly Asp Lys Phe Ile Ile Tyr Asp Ser Ser Phe Asp Asp Ala Asn Arg
 1380 1385 1390

ttc aac ttg gtc cca ctc ttc aag ttc ggc aag gat gag aac tct gat 4223

Phe Asn Leu Val Pro Leu Phe Lys Phe Gly Lys Asp Glu Asn Ser Asp	
1395 1400 1405	
gac tcc atc tgc atc tac aac gag aac cca agc tct gag gac aag aag	4271
Asp Ser Ile Cys Ile Tyr Asn Glu Asn Pro S r Ser Glu Asp Lys Lys	
1410 1415 1420	
tgg tac ttc agc tcc aag gac gac aac aag act gct gac tac aac ggt	4319
Trp Tyr Phe Ser Ser Lys Asp Asp Asn Lys Thr Ala Asp Tyr Asn Gly	
1425 1430 1435	
ggc acc caa tgc att gat gct ggc acc tcc aac aag gac ttc tac tac	4367
Gly Thr Gln Cys Ile Asp Ala Gly Thr Ser Asn Lys Asp Phe Tyr Tyr	
1440 1445 1450 1455	
aac ctc caa gag att gag gtc atc tct gtc act ggt ggc tac tgg tcc	4415
Asn Leu Gln Glu Ile Glu Val Ile Ser Val Thr Gly Gly Tyr Trp Ser	
1460 1465 1470	
agc tac aag atc agc aac ccc atc aac atc aac act ggc att gac tct	4463
Ser Tyr Lys Ile Ser Asn Pro Ile Asn Ile Asn Thr Gly Ile Asp Ser	
1475 1480 1485	
gcc aag gtc aag gtc act gtc aag gct ggt ggc gat gac caa atc ttc	4511
Ala Lys Val Lys Val Thr Val Lys Ala Gly Gly Asp Asp Gln Ile Phe	
1490 1495 1500	
act gct gac aac tcc acc tac gtc cca cag caa cct gct cca tcc ttc	4559
Thr Ala Asp Asn Ser Thr Tyr Val Pro Gln Gln Pro Ala Pro Ser Phe	
1505 1510 1515	
gag gag atg atc tac caa ttc aac aac ctc acc att gac tgc aag aac	4607
Glu Glu Met Ile Tyr Gln Phe Asn Asn Leu Thr Ile Asp Cys Lys Asn	
1520 1525 1530 1535	
ctc aac ttc att gac aac cag gct cac att gag att gac ttc act gcc	4655
Leu Asn Phe Ile Asp Asn Gln Ala His Ile Glu Ile Asp Phe Thr Ala	
1540 1545 1550	
aca gct caa gat ggc cgc ttc ttg ggt gct gag acc ttc atc att cca	4703
Thr Ala Gln Asp Gly Arg Phe Leu Gly Ala Glu Thr Phe Ile Ile Pro	
1555 1560 1565	
gtc acc aag aag gtc ctt ggc act gag aac gtc att gct ctc tac tct	4751
Val Thr Lys Lys Val Leu Gly Thr Glu Asn Val Ile Ala Leu Tyr Ser	
1570 1575 1580	
gag aac aac ggt gtc cag tac atg caa att ggt gct tac aga acc agg	4799
Glu Asn Asn Gly Val Gln Tyr Met Gln Ile Gly Ala Tyr Arg Thr Arg	
1585 1590 1595	
ctc aac acc ctc ttc gct caa cag ttg gtc tcc cgt gcc aac aga ggc	4847
Leu Asn Thr Leu Phe Ala Gln Gln Leu Val Ser Arg Ala Asn Arg Gly	
1600 1605 1610 1615	
att gat gct gtc ctc agc atg gag act cag aac atc caa gag cca caa	4895
Ile Asp Ala Val Leu Ser Met Glu Thr Gln Asn Ile Gln Glu Pro Gln	
1620 1625 1630	
ctt ggt gct ggc acc tac gtc caa ctt gtc ttg gac aag tac gat gag	4943
Leu Gly Ala Gly Thr Tyr Val Gln Leu Val L u Asp Lys Tyr Asp Glu	

1635	1640	1645	
tcc att cat ggc acc aac aag tcc ttc gcc att gag tac gtg gac atc Ser Ile His Gly Thr Asn Lys Ser Phe Ala Ile Glu Tyr Val Asp Ile 1650 1655 1660			4991
ttc aag gag aac gac tcc ttc gtc atc tac caa ggt gag ttg tct gag Phe Lys Glu Asn Asp Ser Phe Val Ile Tyr Gln Gly Glu Leu Ser Glu 1665 1670 1675			5039
acc tcc caa act gtg gtc aag gtc ttc ctc tcc tac ttc att gag gcc Thr Ser Gln Thr Val Val Lys Val Phe Leu Ser Tyr Phe Ile Glu Ala 1680 1685 1690 1695			5087
acc ggt aac aag aac cac ctc tgg gtc agg gcc aag tac cag aag gag Thr Gly Asn Lys Asn His Leu Trp Val Arg Ala Lys Tyr Gln Lys Glu 1700 1705 1710			5135
acc act gac aag atc ctc ttc gac agg act gat gag aag gac cca cat Thr Thr Asp Lys Ile Leu Phe Asp Arg Thr Asp Glu Lys Asp Pro His 1715 1720 1725			5183
ggg tgg ttc ctc tct gat gac cac aag acc ttc tct ggt ctc agc tct Gly Trp Phe Leu Ser Asp Asp His Lys Thr Phe Ser Gly Leu Ser Ser 1730 1735 1740			5231
gct caa gct ctc aag aac gac tct gag cca atg gac ttc tct ggt gcc Ala Gln Ala Leu Lys Asn Asp Ser Glu Pro Met Asp Phe Ser Gly Ala 1745 1750 1755			5279
aac gct ctc tac ttc tgg gag ttg ttc tac tac act cca atg atg atg Asn Ala Leu Tyr Phe Trp Glu Leu Phe Tyr Tyr Thr Pro Met Met Met 1760 1765 1770 1775			5327
gct cac agg ctc ctt caa gag cag aac ttc gat gct gcc aac cac tgg Ala His Arg Leu Leu Gln Glu Gln Asn Phe Asp Ala Ala Asn His Trp 1780 1785 1790			5375
ttc cgc tac gtc tgg agc cca tct ggt tac att gtg gat ggc aag att Phe Arg Tyr Val Trp Ser Pro Ser Gly Tyr Ile Val Asp Gly Lys Ile 1795 1800 1805			5423
gcc atc tac cac tgg aac gtc agg cca ttg gag gag gac acc tcc tgg Ala Ile Tyr His Trp Asn Val Arg Pro Leu Glu Asp Thr Ser Trp 1810 1815 1820			5471
aac gct cag caa ctt gac tcc act gac cca gat gct gtg gct caa gat Asn Ala Gln Gln Leu Asp Ser Thr Asp Pro Asp Ala Val Ala Gln Asp 1825 1830 1835			5519
gac cca atg cac tac aag gtg gcc acc ttc atg gcc acc ttg gac ctt Asp Pro Met His Tyr Lys Val Ala Thr Phe Met Ala Thr Leu Asp Leu 1840 1845 1850 1855			5567
ctc atg gcc aga ggt gat gct gcc tac cgc caa ttg gag agg gac acc Leu Met Ala Arg Gly Asp Ala Ala Tyr Arg Gln Leu Glu Arg Asp Thr 1860 1865 1870			5615
ttg gct gag gcc aag atg tgg tac acc caa gct ctc aac ttg ctg ggt Leu Ala Glu Ala Lys Met Trp Tyr Thr Gln Ala Leu Asn Leu Leu Gly 1875 1880 1885			5663

gat gag cca caa gtc atg ctc tcc aca acc tgg gcc aac cca acc ttg Asp Glu Pro Gln Val Met Leu Ser Thr Thr Trp Ala Asn Pro Thr Leu 1890 1895 1900	5711
ggc aac gct gcc tcc aag acc aca caa cag gtc agg caa cag gtc ctc Gly Asn Ala Ala Ser Lys Thr Thr Gln Gln Val Arg Gln Gln Val Leu 1905 1910 1915	5759
acc caa ctc agg ctc aac tct aga gtc aag act cca ctc ttg ggc act Thr Gln Leu Arg Leu Asn Ser Arg Val Lys Thr Pro Leu Leu Gly Thr 1920 1925 1930 1935	5807
gcc aac tcc ctc act gct ctc ttc ctc cca caa gag aac tcc aaa ctt Ala Asn Ser Leu Thr Ala Leu Phe Leu Pro Gln Glu Asn Ser Lys Leu 1940 1945 1950	5855
aag ggt tac tgg agg acc ctt gct caa cgc atg ttc aac ctc agg cac Lys Gly Tyr Trp Arg Thr Leu Ala Gln Arg Met Phe Asn Leu Arg His 1955 1960 1965	5903
aac ctc tcc att gat ggt caa cca ctc tcc ttg cca ctc tac gct aag Asn Leu Ser Ile Asp Gly Gln Pro Leu Ser Leu Pro Leu Tyr Ala Lys 1970 1975 1980	5951
cca gct gac cca aag gct ctc ctt tcc gct gct gtc tcc gca tcc caa Pro Ala Asp Pro Lys Ala Leu Leu Ser Ala Ala Val Ser Ala Ser Gln 1985 1990 1995	5999
ggt ggt gct gac ctc cca aag gct cca ctc acc atc cac agg ttc cca Gly Gly Ala Asp Leu Pro Lys Ala Pro Leu Thr Ile His Arg Phe Pro 2000 2005 2010 2015	6047
caa atg ttg gag ggt gcc cgt ggt ctt gtc aac cag ctc atc caa ttc Gln Met Leu Glu Gly Ala Arg Gly Leu Val Asn Gln Leu Ile Gln Phe 2020 2025 2030	6095
ggt tcc tct ctc ctt ggt tac tct gag agg caa gat gct gag gcc atg Gly Ser Ser Leu Leu Gly Tyr Ser Glu Arg Gln Asp Ala Glu Ala Met 2035 2040 2045	6143
tcc caa ctc ttg caa acc cag gct tct gag ttg atc ctc acc tcc atc Ser Gln Leu Leu Gln Thr Gln Ala Ser Glu Leu Ile Leu Thr Ser Ile 2050 2055 2060	6191
agg atg caa gac aac cag ctt gct gag ttg gac tct gag aag act gct Arg Met Gln Asp Asn Gln Leu Ala Glu Leu Asp Ser Glu Lys Thr Ala 2065 2070 2075	6239
ctc caa gtc tcc ctt gct ggt gtc caa cag agg ttc gac agc tac tcc Leu Gln Val Ser Leu Ala Gly Val Gln Gln Arg Phe Asp Ser Tyr Ser 2080 2085 2090 2095	6287
caa ctc tac gag gag aac atc aac gct ggt gag caa agg gct ttg gct Gln Leu Tyr Glu Glu Asn Ile Asn Ala Gly Glu Gln Arg Ala Leu Ala 2100 2105 2110	6335
ctc agg tct gag tct gcc att gag tcc caa ggt gct caa atc tcc cgc Leu Arg Ser Glu Ser Ala Ile Glu Ser Gln Gly Ala Gln Ile Ser Arg 2115 2120 2125	6383

atg gct ggt gct ggc gtg gac atg gct cca aac atc ttc ggt ctt gct Met Ala Gly Ala Gly Val Asp Met Ala Pro Asn Ile Phe Gly Leu Ala 2130 2135 2140	6431
gat ggt ggc atg cac tac ggt gcc att gct tac gcc att gct gat ggc Asp Gly Gly Met His Tyr Gly Ala Ile Ala Tyr Ala Ile Ala Asp Gly 2145 2150 2155	6479
att gag ctt tct gct tct gcc aag atg gtt gat gct gag aag gtg gct Ile Glu Leu Ser Ala Ser Ala Lys Met Val Asp Ala Glu Lys Val Ala 2160 2165 2170 2175	6527
caa tct gaa atc tac cgt cgc aga cgc caa gaa tgg aag atc caa agg Gln Ser Glu Ile Tyr Arg Arg Arg Arg Gln Glu Trp Lys Ile Gln Arg 2180 2185 2190	6575
gac aac gct caa gct gag atc aac cag ctc aac gct caa ctt gag tcc Asp Asn Ala Gln Ala Glu Ile Asn Gln Leu Asn Ala Gln Leu Glu Ser 2195 2200 2205	6623
ctc agc atc agg cgt gag gct gct gag atg cag aag gag tac ctc aag Leu Ser Ile Arg Arg Glu Ala Ala Glu Met Gln Lys Glu Tyr Leu Lys 2210 2215 2220	6671
acc caa cag gct caa gct cag gct caa ctc acc ttc ctc agg tcc aag Thr Gln Gln Ala Gln Ala Gln Ala Gln Leu Thr Phe Leu Arg Ser Lys 2225 2230 2235	6719
ttc tcc aac cag gct ctc tac tcc tgg ctc aga ggc cgc ctc tct ggc Phe Ser Asn Gln Ala Leu Tyr Ser Trp Leu Arg Gly Arg Leu Ser Gly 2240 2245 2250 2255	6767
atc tac ttc caa ttc tac gac ttg gct gtc tcc cgc tgc ctc atg gct Ile Tyr Phe Gln Phe Tyr Asp Leu Ala Val Ser Arg Cys Leu Met Ala 2260 2265 2270	6815
gag caa tcc tac caa tgg gag gcc aac gac aac agc atc tcc ttc gtc Glu Gln Ser Tyr Gln Trp Glu Ala Asn Asp Asn Ser Ile Ser Phe Val 2275 2280 2285	6863
aag cca ggt gct tgg caa ggc acc tac gct ggt ctc ctt tgc ggt gag Lys Pro Gly Ala Trp Gln Gly Thr Tyr Ala Gly Leu Leu Cys Gly Glu 2290 2295 2300	6911
gct ctc atc cag aac ttg gct caa atg gag gag gct tac ctc aag tgg Ala Leu Ile Gln Asn Leu Ala Gln Met Glu Glu Ala Tyr Leu Lys Trp 2305 2310 2315	6959
gag tcc aga gct ttg gag gta gag agg act gtc tcc ctt gct gta gtc Glu Ser Arg Ala Leu Glu Val Glu Arg Thr Val Ser Leu Ala Val Val 2320 2325 2330 2335	7007
tac gac tcc ttg gag ggc aac gac agg ttc aac ctt gct gag caa atc Tyr Asp Ser Leu Glu Gly Asn Asp Arg Phe Asn Leu Ala Glu Gln Ile 2340 2345 2350	7055
cca gct ctc ttg gac aag ggt gag ggc act gct ggc acc aag gag aac Pro Ala Leu Leu Asp Lys Gly Glu Gly Thr Ala Gly Thr Lys Glu Asn 2355 2360 2365	7103
ggt ctc tcc ttg gcc aac gcc atc ctc tct gct tct gtc aag ctc tct	7151

Gly Leu Ser Leu Ala Asn Ala Ile Leu Ser Ala Ser Val Lys Leu Ser
 2370 2375 2380

gac ctc aag ttg ggt act gac tac cca gac tcc att gtg ggt tcc aac 7199
 Asp Leu Lys Leu Gly Thr Asp Tyr Pro Asp Ser Ile Val Gly Ser Asn
 2385 2390 2395

aag gtc aga agg atc aag caa atc tct gtc tcc ctc cca gct ttg gtg 7247
 Lys Val Arg Arg Ile Lys Gln Ile Ser Val Ser Leu Pro Ala Leu Val
 2400 2405 2410 2415

ggt cca tac caa gat gtc caa gcc atg ctc tcc tac ggt ggc tcc acc 7295
 Gly Pro Tyr Gln Asp Val Gln Ala Met Leu Ser Tyr Gly Gly Ser Thr
 2420 2425 2430

caa ctc cca aag ggt tgc tct gct ttg gct gtc tcc cac ggc acc aac 7343
 Gln Leu Pro Lys Gly Cys Ser Ala Leu Ala Val Ser His Gly Thr Asn
 2435 2440 2445

gac tct ggt caa ttc caa ctt gac ttc aac gat ggc aag tac ctc cca 7391
 Asp Ser Gly Gln Phe Gln Leu Asp Phe Asn Asp Gly Lys Tyr Leu Pro
 2450 2455 2460

ttc gaa ggc att gct ttg gat gac caa ggc acc ctc aac ctc caa ttc 7439
 Phe Glu Gly Ile Ala Leu Asp Asp Gln Gly Thr Leu Asn Leu Gln Phe
 2465 2470 2475

cca aac gcc act gac aag cag aag gcc atc ctc caa acc atg tct gac 7487
 Pro Asn Ala Thr Asp Lys Gln Lys Ala Ile Leu Gln Thr Met Ser Asp
 2480 2485 2490 2495

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 Ile Ile Leu His Ile Arg Tyr Thr Ile Arg
 2500 2505

ccgc 7541

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 <213> Artificial Sequence

<220>
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 encoding ER signal from 15 kDa zein from Black
 Mexican Sweet maize

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 Ala Cys Ala Ser Ala
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<210> 6

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<212> DNA

<213> Artificial Sequence

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fused to the modified 15 kDa zein endoplasmic
reticulum signal peptide

<220>

<221> CDS

<222> (4)..(7614)

<400> 6

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gct gcc tgt gct tca gcc atg aac gag tcc gtc aag gag atc cca gac   96
Ala Ala Cys Ala Ser Ala Met Asn Glu Ser Val Lys Glu Ile Pro Asp
          20             25             30

gtc ctc aag tcc caa tgc ggt ttc aac tgc ctc act gac atc tcc cac   144
Val Leu Lys Ser Gln Cys Gly Phe Asn Cys Leu Thr Asp Ile Ser His
          35             40             45

agc tcc ttc aac gag ttc aga caa caa gtc tct gag cac ctc tcc tgg   192
Ser Ser Phe Asn Glu Phe Arg Gln Gln Val Ser Glu His Leu Ser Trp
          50             55             60

tcc gag acc cat gac ctc tac cat gac gct cag caa gct cag aag gac   240
Ser Glu Thr His Asp Leu Tyr His Asp Ala Gln Gln Ala Gln Lys Asp
          65             70             75

aac agg ctc tac gag gct agg atc ctc aag agg gct aac cca caa ctc   288
Asn Arg Leu Tyr Glu Ala Arg Ile Leu Lys Arg Ala Asn Pro Gln Leu
          80             85             90             95

cag aac gct gtc cac ctc gcc atc ttg gct cca aac gct gag ttg att   336
Gln Asn Ala Val His Leu Ala Ile Leu Ala Pro Asn Ala Glu Leu Ile
          100            105            110

ggt tac aac aac cag ttc tct ggc aga gct agc cag tac gtg gct cct   384
Gly Tyr Asn Asn Gln Phe Ser Gly Arg Ala Ser Gln Tyr Val Ala Pro
          115            120            125

ggt aca gtc tcc tcc atg ttc agc cca gcc gct tac ctc act gag ttg   432
Gly Thr Val Ser Ser Met Phe Ser Pro Ala Ala Tyr Leu Thr Glu Leu
          130            135            140

tac cgc gag gct agg aac ctt cat gct tct gac tcc gtc tac tac ttg   480
Tyr Arg Glu Ala Arg Asn Leu His Ala Ser Asp Ser Val Tyr Tyr Leu
          145            150            155

gac aca cgc aga cca gac ctc aag agc atg gcc ctc agc caa cag aac   528
Asp Thr Arg Arg Pro Asp Leu Lys Ser Met Ala Leu Ser Gln Gln Asn
          160            165            170            175

atg gac att gag ttg tcc acc ctc tcc ttg agc aac gag ctt ctc ttg   576

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180 185 190	
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Glu Ser Ile Lys Thr Glu Ser Lys Leu Glu Asn Tyr Thr Lys Val Met	
195 200 205	
gag atg ctc tcc acc ttc aga cca agc ggt gca act cca tac cat gat	672
Glu Met Leu Ser Thr Phe Arg Pro Ser Gly Ala Thr Pro Tyr His Asp	
210 215 220	
gcc tac gag aac gtc agg gag gtc atc caa ctt caa gac cct ggt ctt	720
Ala Tyr Glu Asn Val Arg Glu Val Ile Gln Leu Gln Asp Pro Gly Leu	
225 230 235	
gag caa ctc aac gct tct cca gcc att gct ggt ttg atg cac cag gca	768
Glu Gln Leu Asn Ala Ser Pro Ala Ile Ala Gly Leu Met His Gln Ala	
240 245 250 255	
tcc ttg ctc ggt atc aac gcc tcc atc tct cct gag ttg ttc aac atc	816
Ser Leu Leu Gly Ile Asn Ala Ser Ile Ser Pro Glu Leu Phe Asn Ile	
260 265 270	
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Leu Thr Glu Glu Ile Thr Glu Gly Asn Ala Glu Glu Leu Tyr Lys Lys	
275 280 285	
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Asn Phe Gly Asn Ile Glu Pro Ala Ser Leu Ala Met Pro Glu Tyr Leu	
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Lys Arg Tyr Tyr Asn Leu Ser Asp Glu Glu Leu Ser Gln Phe Ile Gly	
305 310 315	
aag gct tcc aac ttc ggt caa cag gag tac agc aac aac cag ctc atc	1008
Lys Ala Ser Asn Phe Gly Gln Gln Glu Tyr Ser Asn Asn Gln Leu Ile	
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Thr Pro Val Val Asn Ser Ser Asp Gly Thr Val Lys Val Tyr Arg Ile	
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Thr Arg Glu Tyr Thr Thr Asn Ala Tyr Gln Met Asp Val Glu Leu Phe	
355 360 365	
cca ttc ggt ggt gag aac tac aga ctt gac tac aag ttc aag aac ttc	1152
Pro Phe Gly Gly Glu Asn Tyr Arg Leu Asp Tyr Lys Phe Lys Asn Phe	
370 375 380	
tac aac gcc tcc tac ctc tcc atc aag ttg aac gac aag agg gag ctt	1200
Tyr Asn Ala Ser Tyr Leu Ser Ile Lys Leu Asn Asp Lys Arg Glu Leu	
385 390 395	
gtc agg act gag ggt gct cct caa gtg aac att gag tac tct gcc aac	1248
Val Arg Thr Glu Gly Ala Pro Gln Val Asn Ile Glu Tyr Ser Ala Asn	
400 405 410 415	
atc acc ctc aac aca gct gac atc tct caa cca ttc gag att ggt ttg	1296
Ile Thr Leu Asn Thr Ala Asp Ile Ser Gln Pro Phe Glu Ile Gly Leu	

46

ctc ttc atc atg acc agc acc tcc tac aac aag acc ctc act cct gag	2064
Leu Phe Ile Met Thr Ser Thr Ser Tyr Asn Lys Thr Leu Thr Pro Glu	
675 680 685	
atc aag aac ctc ttg gac aca gtc tac cac ggt ctc caa ggc ttc gac	2112
Ile Lys Asn Leu Leu Asp Thr Val Tyr His Gly Leu Gln Gly Phe Asp	
690 695 700	
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Lys Asp Lys Ala Asp Leu Leu His Val Met Ala Pro Tyr Ile Ala Ala	
705 710 715	
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Thr Leu Gln Leu Ser Ser Glu Asn Val Ala His Ser Val Leu Leu Trp	
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Ala Asp Lys Leu Gln Pro Gly Asp Gly Ala Met Thr Ala Glu Lys Phe	
740 745 750	
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Trp Asp Trp Leu Asn Thr Lys Tyr Thr Pro Gly Ser Ser Glu Ala Val	
755 760 765	
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Glu Thr Gln Glu His Ile Val Gln Tyr Cys Gln Ala Leu Ala Gln Leu	
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785 790 795	
ttc gtc acc aag cct gag atg ttc ggt gct gcc aca ggt gct gca cct	2448
Phe Val Thr Lys Pro Glu Met Phe Gly Ala Thr Gly Ala Ala Pro	
800 805 810 815	
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Ala His Asp Ala Leu Ser Leu Ile Met Leu Thr Arg Phe Ala Asp Trp	
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Val Asn Ala Leu Gly Glu Lys Ala Ser Ser Val Leu Ala Ala Phe Glu	
835 840 845	
gcc aac tcc ctc act gct gag caa ctt gct gat gcc atg aac ctt gat	2592
Ala Asn Ser Leu Thr Ala Glu Gln Leu Ala Asp Ala Met Asn Leu Asp	
850 855 860	
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Ala Asn Leu Leu Leu Gln Ala Ser Ile Gln Ala Gln Asn His Gln His	
865 870 875	
ctc cca cct gtc act cca gag aac gct ttc tcc tgc tgg acc tcc atc	2688
Leu Pro Pro Val Thr Pro Glu Asn Ala Phe Ser Cys Trp Thr Ser Ile	
880 885 890 895	
aac acc atc ctc caa tgg gtc aac gtg gct cag caa ctc aac gtg gct	2736
Asn Thr Ile Leu Trp Val Asn Val Ala Gln Gln Leu Asn Val Ala	
900 905 910	

cca caa ggt gtc tct gct ttg gtc ggt ctt gac tac atc cag tcc atg	2784
Pro Gln Gly Val Ser Ala Leu Val Gly Leu Asp Tyr Ile Gln Ser Met	
915 920 925	
aag gag aca cca acc tac gct caa tgg gag aac gca gct ggt gtc ttg	2832
Lys Glu Thr Pro Thr Tyr Ala Gln Trp Glu Asn Ala Ala Gly Val Leu	
930 935 940	
act gct ggt ctc aac tcc caa cag gcc aac acc ctc cat gct ttc ttg	2880
Thr Ala Gly Leu Asn Ser Gln Gln Ala Asn Thr Leu His Ala Phe Leu	
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gat gag tct cgc tct gct gcc ctc tcc acc tac tac atc agg caa gtc	2928
Asp Glu Ser Arg Ser Ala Ala Leu Ser Thr Tyr Tyr Ile Arg Gln Val	
960 965 970 975	
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980 985 990	
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Leu Leu Ile Asp Asn Gln Val Ser Ala Ala Ile Lys Thr Thr Arg Ile	
995 1000 1005	
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Asn Val Glu Glu Asn Ala Asn Ser Gly Val Ile Ser Arg Gln Phe Phe	
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Ser Gln Leu Val Tyr Tyr Pro Glu Asn Tyr Ile Asp Pro Thr Met Arg	
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Ser Gln Leu Asn Ala Asp Thr Val Glu Asp Ala Phe Met Ser Tyr Leu	
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Thr Ser Phe Glu Gln Val Ala Asn Leu Lys Val Ile Ser Ala Tyr His	
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Asp Asn Ile Asn Asn Asp Gln Gly Leu Thr Tyr Phe Ile Gly Leu Ser	
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Ile Asp Cys Pro Ile Asn Pro Tyr Lys Ser Thr Ile Arg Pro Val Ile	
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tac aag agc cgc ctc tac ttg ctc tgg ctt gag cag aag gag atc acc	3600
Tyr Lys Ser Arg Leu Tyr Leu Leu Trp Leu Glu Gln Lys Glu Ile Thr	
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Lys Gln Thr Gly Asn Ser Lys Asp Gly Tyr Gln Thr Glu Thr Asp Tyr	
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cgc tac gag ttg aag ttg gct cac atc cgc tac gat ggt acc tgg aac	3696
Arg Tyr Glu Leu Lys Leu Ala His Ile Arg Tyr Asp Gly Thr Trp Asn	
1220 1225 1230	
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Thr Pro Ile Thr Phe Asp Val Asn Lys Lys Ile Ser Glu Leu Lys Leu	
1235 1240 1245	
gag aag aac cgt gct cct ggt ctc tac tgc gct ggt tac caa ggt gag	3792
Glu Lys Asn Arg Ala Pro Gly Leu Tyr Cys Ala Gly Tyr Gln Gly Glu	
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gac acc ctc ttg gtc atg ttc tac aac cag caa gac acc ctt gac tcc	3840
Asp Thr Leu Leu Val Met Phe Tyr Asn Gln Gln Asp Thr Leu Asp Ser	
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Tyr Lys Asn Ala Ser Met Gln Gly Leu Tyr Ile Phe Ala Asp Met Ala	
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Ser Lys Asp Met Thr Pro Glu Gln Ser Asn Val Tyr Arg Asp Asn Ser	
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Tyr Gln Gln Phe Asp Thr Asn Asn Val Arg Arg Val Asn Asn Arg Tyr	
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acc atc aac tac aag gct gcc tct tcc gac ctc aaa atc tac atc agc	4128
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Pro Lys Leu Arg Ile Ile His Asn Gly Tyr Glu Gly Gln Lys Arg Asn	
1380 1385 1390	
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Gln Cys Asn Leu Met Asn Lys Tyr Gly Lys Leu Gly Asp Lys Phe Ile	

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Val Tyr Thr Ser Leu Gly Val Asn Pro Asn Asn Ser Ser Asn Lys Leu			
1410	1415	1420	
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Met Phe Tyr Pro Val Tyr Gln Tyr Ser Gly Asn Thr Ser Gly Leu Asn			
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cag ggt aga ctc ttg ttc cac agg gac acc acc tac cca agc aag gtg			4368
Gln Gly Arg Leu Leu Phe His Arg Asp Thr Thr Tyr Pro Ser Lys Val			
1440	1445	1450	1455
gag gct tgg att cct ggt gcc aag agg tcc ctc acc aac cag aac gct			4416
Glu Ala Trp Ile Pro Gly Ala Lys Arg Ser Leu Thr Asn Gln Asn Ala			
1460	1465	1470	
gcc att ggt gat gac tac gcc aca gac tcc ctc aac aag cct gat gac			4464
Ala Ile Gly Asp Asp Tyr Ala Thr Asp Ser Leu Asn Lys Pro Asp Asp			
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Leu Lys Gln Tyr Ile Phe Met Thr Asp Ser Lys Gly Thr Ala Thr Asp			
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gtc tct ggt cca gtg gag atc aac act gca atc agc cca gcc aag gtc			4560
Val Ser Gly Pro Val Glu Ile Asn Thr Ala Ile Ser Pro Ala Lys Val			
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Gln Ile Ile Val Lys Ala Gly Gly Lys Glu Gln Thr Phe Thr Ala Asp			
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Lys Asp Val Ser Ile Gln Pro Ser Pro Ser Phe Asp Glu Met Asn Tyr			
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Gln Phe Asn Ala Leu Glu Ile Asp Gly Ser Gly Leu Asn Phe Ile Asn			
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aac tct gct tcc att gat gtc acc ttc act gcc ttc gct gag gat ggc			4752
Asn Ser Ala Ser Ile Asp Val Thr Phe Thr Ala Phe Ala Glu Asp Gly			
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Arg Lys Leu Gly Tyr Glu Ser Phe Ser Ile Pro Val Thr Leu Lys Val			
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Ser Thr Asp Asn Ala Leu Thr Leu His His Asn Glu Asn Gly Ala Gln			
1600	1605	1610	1615
tac atg caa tgg caa agc tac cgc acc agg ttg aac acc ctc ttc gca			4896
Tyr Met Gln Trp Gln Ser Tyr Arg Thr Arg Leu Asn Thr Leu Phe Ala			
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agg caa ctt gtg gcc cgt gcc acc aca ggc att gac acc atc ctc agc			4944
Arg Gln Leu Val Ala Arg Ala Thr Thr Gly Ile Asp Thr Ile Leu Ser			
1635	1640	1645	

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Met Glu Thr Gln Asn Ile Gln Glu Pro Gln Leu Gly Lys Gly Phe Tyr	
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Ala Thr Phe Val Ile Pro Pro Tyr Asn Leu Ser Thr His Gly Asp Glu	
1665 1670 1675	
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Pro His Phe Val Arg Asp Asp Lys Gly Ile Val Thr Ile Asn Pro Lys	
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Ser Gly Tyr Ile Val His Gly Gln Ile Gln Asn Tyr Gln Trp Asn Val	
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Val Asp Pro Asp Ala Val Ala Gln His Asp Pro Met His Tyr Lys Val	
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Ser Thr Phe Met Arg Thr Leu Asp Leu Leu Ile Ala Arg Gly Asp His	
1875 1880 1885	

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 1890 1895 1900

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 1940 1945 1950

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 1955 1960 1965

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 1970 1975 1980

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 1985 1990 1995

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 Gln Pro Leu Tyr Leu Pro Ile Tyr Ala Thr Pro Ala Asp Pro Lys Ala
 2000 2005 2010 2015

ctt ctc tct gct gct gtg gct acc agc caa ggt ggt ggc aag ctc cca 6096
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 2020 2025 2030

gag tcc ttc atg tcc ctc tgg agg ttc cca cac atg ttg gag aac gcc 6144
 Glu Ser Phe Met Ser Leu Trp Arg Phe Pro His Met Leu Glu Asn Ala
 2035 2040 2045

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 Arg Gly Met Val Ser Gln Leu Thr Gln Phe Gly Ser Thr Leu Gln Asn
 2050 2055 2060

atc att gag agg caa gat gct gag gct ctc aac gct ttg ctc cag aac 6240
 Ile Ile Glu Arg Gln Asp Ala Glu Ala Leu Asn Ala Leu Leu Gln Asn
 2065 2070 2075

cag gca gct gag ttg atc ctc acc aac ttg tcc atc caa gac aag acc 6288
 Gln Ala Ala Glu Leu Ile Leu Thr Asn Leu Ser Ile Gln Asp Lys Thr
 2080 2085 2090 2095

att gag gag ctt gat gct gag aag aca gtc ctt gag aag agc aag gct 6336
 Ile Glu Glu Leu Asp Ala Glu Lys Thr Val Leu Glu Lys Ser Lys Ala
 2100 2105 2110

ggt gcc caa tct cgc ttc gac tcc tac ggc aag ctc tac gat gag aac 6384
 Gly Ala Gln Ser Arg Phe Asp Ser Tyr Gly Lys Leu Tyr Asp Glu Asn
 2115 2120 2125

atc aac gct ggt gag aac cag gcc atg acc ctc agg gct tcc gca gct 6432

Ile Asn Ala Gly Glu Asn Gln Ala Met Thr Leu Arg Ala Ser Ala Ala	
2130 2135 2140	
ggt ctc acc act gct gtc caa gcc tct cgc ttg gct ggt gca gct gct	6480
Gly Leu Thr Thr Ala Val Gln Ala Ser Arg Leu Ala Gly Ala Ala Ala	
2145 2150 2155	
gac ctc gtt cca aac atc ttc ggt ttc gct ggt ggt ggc tcc aga tgg	6528
Asp Leu Val Pro Asn Ile Phe Gly Phe Ala Gly Gly Gly Ser Arg Trp	
2160 2165 2170 2175	
ggt gcc att gct gag gct acc ggt tac gtc atg gag ttc tct gcc aac	6576
Gly Ala Ile Ala Glu Ala Thr Gly Tyr Val Met Glu Phe Ser Ala Asn	
2180 2185 2190	
gtc atg aac act gag gct gac aag atc agc caa tct gag acc tac aga	6624
Val Met Asn Thr Glu Ala Asp Lys Ile Ser Gln Ser Glu Thr Tyr Arg	
2195 2200 2205	
agg cgc cgt caa gag tgg gag atc caa agg aac aac gct gag gca gag	6672
Arg Arg Arg Gln Glu Trp Glu Ile Gln Arg Asn Asn Ala Glu Ala Glu	
2210 2215 2220	
ttg aag caa atc gat gct caa ctc aag tcc ttg gct gtc aga agg gag	6720
Leu Lys Gln Ile Asp Ala Gln Leu Lys Ser Leu Ala Val Arg Arg Glu	
2225 2230 2235	
gct gct gtc ctc cag aag acc tcc ctc aag acc caa cag gag caa acc	6768
Ala Ala Val Leu Gln Lys Thr Ser Leu Lys Thr Gln Gln Glu Gln Thr	
2240 2245 2250 2255	
cag tcc cag ttg gct ttc ctc caa agg aag ttc tcc aac cag gct ctc	6816
Gln Ser Gln Leu Ala Phe Leu Gln Arg Lys Phe Ser Asn Gln Ala Leu	
2260 2265 2270	
tac aac tgg ctc aga ggc cgc ttg gct gcc atc tac ttc caa ttc tac	6864
Tyr Asn Trp Leu Arg Gly Arg Leu Ala Ala Ile Tyr Phe Gln Phe Tyr	
2275 2280 2285	
gac ctt gct gtg gcc agg tgc ctc atg gct gag caa gcc tac cgc tgg	6912
Asp Leu Ala Val Ala Arg Cys Leu Met Ala Glu Gln Ala Tyr Arg Trp	
2290 2295 2300	
gag ttg aac gat gac tcc gcc agg ttc atc aag cca ggt gct tgg caa	6960
Glu Leu Asn Asp Asp Ser Ala Arg Phe Ile Lys Pro Gly Ala Trp Gln	
2305 2310 2315	
ggc acc tac gct ggt ctc ctt gct ggt gag acc ctc atg ctc tcc ttg	7008
Gly Thr Tyr Ala Gly Leu Leu Ala Gly Glu Thr Leu Met Leu Ser Leu	
2320 2325 2330 2335	
gct caa atg gag gat gct cac ctc aag agg gac aag agg gct ttg gag	7056
Ala Gln Met Glu Asp Ala His Leu Lys Arg Asp Lys Arg Ala Leu Glu	
2340 2345 2350	
gtg gag agg aca gtc tcc ctt gct gag gtc tac gct ggt ctc cca aag	7104
Val Glu Arg Thr Val Ser Leu Ala Glu Val Tyr Ala Gly Leu Pro Lys	
2355 2360 2365	
gac aac ggt cca ttc tcc ctt gct caa gag att gac aag ttg gtc agc	7152
Asp Asn Gly Pro Phe Ser Leu Ala Gln Glu Ile Asp Lys Leu Val Ser	

2370	2375	2380	
caa ggt tct ggt tct gct ggt tct ggt aac aac aac ttg gct ttc ggc			7200
Gln Gly Ser Gly Ser Ala Gly Ser Gly Asn Asn Asn Leu Ala Phe Gly			
2385	2390	2395	
gct ggt act gac acc aag acc tcc ctc caa gcc tct gtc tcc ttc gct			7248
Ala Gly Thr Asp Thr Lys Thr Ser Leu Gln Ala Ser Val Ser Phe Ala			
2400	2405	2410	2415
gac ctc aag atc agg gag gac tac cca gct tcc ctt ggc aag atc agg			7296
Asp Leu Lys Ile Arg Glu Asp Tyr Pro Ala Ser Leu Gly Lys Ile Arg			
	2420	2425	2430
cgc atc aag caa atc tct gtc acc ctc cca gct ctc ttg ggt cca tac			7344
Arg Ile Lys Gln Ile Ser Val Thr Leu Pro Ala Leu Leu Gly Pro Tyr			
	2435	2440	2445
caa gat gtc caa gca atc ctc tcc tac ggt gac aag gct ggt ttg gcg			7392
Gln Asp Val Gln Ala Ile Leu Ser Tyr Gly Asp Lys Ala Gly Leu Ala			
	2450	2455	2460
aac ggt tgc gag gct ctt gct gtc tct cat ggc atg aac gac tct ggt			7440
Asn Gly Cys Glu Ala Leu Ala Val Ser His Gly Met Asn Asp Ser Gly			
	2465	2470	2475
caa ttc caa ctt gac ttc aac gat ggc aag ttc ctc cca ttc gag ggc			7488
Gln Phe Gln Leu Asp Phe Asn Asp Gly Lys Phe Leu Pro Phe Glu Gly			
	2480	2485	2490
att gcc att gac caa ggc acc ctc acc ctc tcc ttc cca aac gct tcc			7536
Ile Ala Ile Asp Gln Gly Thr Leu Thr Leu Ser Phe Pro Asn Ala Ser			
	2500	2505	2510
atg cca gag aag gga aag caa gcc acc atg ctc aag acc ctc aac gat			7584
Met Pro Glu Lys Gly Lys Gln Ala Thr Met Leu Lys Thr Leu Asn Asp			
	2515	2520	2525
atc atc ctc cac atc agg tac acc atc aag tgagctc			7621
Ile Ile Leu His Ile Arg Tyr Thr Ile Lys			
	2530	2535	

INTERNATIONAL SEARCH REPORT

 Interna pplication No
 PCT/US 00/22237

 A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12N9/52 C12N15/82 C07K14/24 C12N15/11

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

 Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

STRAND, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 08932 A (DOW AGROSCIENCES LLC ; WISCONSIN ALUMNI RES FOUND (US)) 5 March 1998 (1998-03-05) cited in the application SEQ ID NO:11 in this document is the unmodified version of SEQ ID NO:3 of the present application. SEQ ID NO:46 corresponds to SEQ ID NO:5. page 16, line 31 -page 19, line 35	1-7
A	WO 97 13402 A (DOWELANCO) 17 April 1997 (1997-04-17) the whole document	1-7

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

1 December 2000

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08/12/2000

Name and mailing address of the ISA

 European Patent Office, P.B. 5818 Patentlaan 2
 NL - 2280 HV Rijswijk
 Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
 Fax: (+31-70) 340-3016

Authorized officer

Sprinks, M

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal application No

PCT/US 00/22237

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